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INDUSTRIAL MICROBIOLOGY

The Utilization of
Bacteria, Yeasts and Molds
in Industrial Processes

INDUSTRIAL MICROBIOLOGY

The Utilization of Bacteria, Yeasts and Molds in Industrial Processes

BY

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PREFACE

In recent years the manufacturer and the industrial chemist have come to appreciate more fully the value of the bacteriologist in industry and to depend more and more on his judgment and advice. Recognizing the growing importance of microbiological processes in industry and the fact that there is no publication devoted primarily to this phase of applied mycology this volume attempts to bring together what information is available in various scattered sources, usually rather meagre and incomplete, and to add thereto such material from the experiences of the authors as teachers and investigators as may be of service to students planning to enter this field or to technical workers already engaged in some phase of the work.

A number of the processes here considered are protected by government patents and in such cases the various patents are referred to and their salient points discussed. Of course students and others will realize that patented processes cannot be employed industrially except by permission of the patent holder, but a study and clear presentation of the patent claims and a discussion of the processes involved will nevertheless be of value to workers in similar or allied lines.

Emphasis will be placed primarily on the microörganismal phase of the process, the source of the organisms involved, methods of obtaining them and of controlling their proper functioning in plant scale operations. Purely chemical phases of the processes will not be considered in detail but the accompanying bibliography may be used as a guide to the sources of such information.

No attempt has been made to make the bibliographies complete, only those articles that seemed to have important practical bearing on the particular problems under discussion being drawn upon, and unnecessary duplication was avoided. Some of the problems considered are discussed in much more detail than others, but we have attempted to select for detailed discussion those fermentations and those phases that we felt would be of particular interest and especially helpful to those engaging in industrial production work of the same or a similar nature.

Review of the literature and discussion of the same has been taken

CHANGES IN VISCOSITY

Microörganisms may increase the viscosity of the substratum as demonstrated in the growth of *Achromobacter viscosum* in milk. The viscosity may be reduced by the partial digestion of protein material, as gelatin and casein, or in the decomposition of starch paste or agar agar.

SHAKING

Bacteria are prevented from multiplying and later killed by shaking. The chemist recognizes the ability of some proteins to be coagulated by long shaking. The effect may be similar in the case of the microorganisms.

TABLE 1

INDICATORS FOR GENERAL USE

INDICATOR	pH RANGE	COLOR CHANGE
Thymol Blue	1.2– 2.8	Red–Yellow
Topfer's reagent	2.9– 4.0	Red–Yellow
Bromphenol Blue	3.0– 4.6	Yellow–Blue
Methyl Red	4.2– 6.3	Red–Yellow
Chlorphenol Red	5.0– 6.6	Yellow–Red
Bromcresol Purple	5.4– 7.0	Yellow–Purple
Bromthymol Blue	6.0– 7.6	Yellow–Blue
Phenol Red	6.6– 8.2	Yellow–Red
Cresol Red	7.2– 8.8	Yellow–Red
Thymol Blue	8.2– 9.8	Yellow–Blue
Phenolphthalein	8.3–10.0	Colorless–Red
Alizarin Yellow	10.0–12.0	Colorless–Yellow

PRESSURE

There have been numerous attempts to sterilize organic substances by applying great pressure to the material for varying periods of time. A process (1) patented in 1920, for destroying cellular structure by exerting positive, negative or alternating pressures, fails to state what the respective pressures may be to destroy the bacteria, "animalculae, like vinegar eels, rotifers, amoeba, parameciidae, etc.," "by explosion from the inside," and "presumably even the embryonic cells." We have found the use of alternating pressure a very satisfactory method of sterilizing; whereas 3000 atmospheres of positive pressure has not killed cultures of *B. coli* a much lower alternating pressure will insure

sterility. Other non-spore forming rods require a pressure of about 6000 atmospheres for a period of fourteen hours before they are destroyed, while spore forms are not uniformaly killed even at 12,000 atmospheres.

Bart, in 1917, patented (2) a process of sterilizing animal and vegetable products by destroying or retarding "the vital functions of microörganisms, ferments, enzyms, and similar bodies" by treating them "with protoxid of nitrogen at a pressure exceeding about five atmospheres and at a temperature which is below twenty-five atmospheres."

ADSORPTION

Bacteria are negatively charged and are not precipitated by small amounts of electrolyte. They resemble the emulsoid type of colloid. When a suspension of bacteria exhibits the agglutination phenomenon the bacterial clump is negatively charged.

REFRACTIVE INDEX

The refractive index of the vegetative cell is lower than that of the spore form and also that of the flagella. The ectoplasm shows a higher refractive index than does the endoplasm.

SPECIFIC GRAVITY

The specific gravity of the various organisms as reported by Rubner (3) is as follows:

Gelatin liquefyers	1.0651
Gas formers	1.0465
Culture from potato	1.0380
M. prodigiosus	1.0540

WEIGHT OF BACTERIA

The weight of bacteria can be readily determined from their size and density. Among the known microörganisms the bacilli far outnumber other forms and are probably the most common variety of bacteria in existence. The bacilli and the variations from this fundamental type may be considered as cylinders.

Assuming the average bacterial cell to be 2 microns long and 0.5 micron in diameter, the weight of the specific cell would be 0.000,000,000,412 mgm., that is, it would require approximately

2,420,000,000,000 such cells to weigh a gram. Specific gravity taken as 1.050.

OXYGEN

The microörganisms which obtain oxygen directly from the atmosphere, in the form of free O_2, constitute a large part of the known species. Chromogenic bacteria belong to this group. Other species are able to obtain energy by reducing processes and they obtain their supply of oxygen indirectly. The most active group is referred to as facultative and grows either under aerobic or anaerobic conditions. Distinct from these facultative forms are the microaerophiles which grow best under reduced oxygen tension, as can be readily ascertained by inoculating deep columns of broth in tubes and carefully incubating without agitation for twenty-four hours and noting the cloudy region. The presence of carbohydrates usually facilitates anaerobiosis although the strict anaerobes are able to obtain their oxygen supply from complex proteins.

TEMPERATURE

The external temperature is related to the metabolism of the microbial cell. Bacteria are generally regarded as poikilothermic, that is, they take the temperature of their environment. While some phosphorescent bacteria grow below 10°C. the mesophilic and thermophilic bacteria are the most useful in industrial processes. The mesophilic range may be regarded as between 20° and 40°C. and the thermophilic range above 40°C., although the true thermophiles are most active at about 55°C. The terminology is not so important as it is generally more efficacious to adjust the external temperature in relation to the specific fermentation. The optimum temperature for growth may not necessarily be the optimum for the production of specific end products. Thermophilic fermentations tend to purify themselves and are not as easily contaminated as those at lower temperatures.

METABIOSIS

In bacteriology metabiosis is the equivalent of the biologists mutual aid. If a fermentation is allowed to proceed unmolested numerous types of microörganisms will decompose the substratum to ultimate gas, water and mineral residue. The struggle for existence of the bacteriologist's strain of the "fittest" type of microörganisms

depends upon the expert preparation and control of the fermenting mash. All of the products formed are suitable nutrient for further microbiological fermentation. The alcohol produced by the yeasts is oxidized by the bacteria, Acetobacters, and the acetic acid in turn is attacked by the molds.

INVOLUTION FORMS

Involution forms of microörganisms are generally designated by a marked change in the size, or more particularly, the shape of a given species from that of the commonly observed form. This "epitaph" does not necessarily mean that the morphology or physiology of the cell is not normal but rather indicates that the cell has adapted itself to its environment. Multiformity, or pleomorphism, is more pronounced in certain species of the bacteria than in other microörganisms. A study of the effect of temperature upon the growing culture readily demonstrates this change, although any change in the composition of the medium may alter the morphology. In the study of cultures used as industrial starters morphology is a very valuable criterion but a knowledge of other attributes is necessary to insure the proper organism.

PURE CULTURES

Pure cultures are not always desirable in industrial processes. Mixed fermentation produces different end products from that obtained, oftentimes, in pure culture and a change in the rate of decomposition with an increase in yield is sometimes the case.

Predominating types are many times the best that can be hoped for, particularly for fermentations in the mesophilic range. Thermophiles are more easily controlled and produce better yields in shorter periods of time. Temperature must often be artificially maintained but the economy of time required will balance the cost of an extraneous source of heat.

ISOLATION OF ORGANISMS

The development of microörganisms upon artificial culture media depends upon the available nutrients, the source of oxygen, the amount of moisture, the proper hydrogen ion range, a suitable temperature for incubation and the preparation of a sterile medium to prevent contamination. Where possible it is generally preferable

to incorporate in the medium varying amounts (1 to 10 per cent) of the substrate to be acted upon in the large scale fermentation. The additions of vegetable tissues and plant tissue extracts are often capable of stimulating growth. In the isolation of the causative agent in an unknown fermentation process it is, again, profitable to utilize the substrate in a solid or liquid medium depending upon the particular case. Generally liquid media are to be preferred for isolating unknown cultures. Transfer to solid media can subsequently be performed as deemed necessary. Information as to the type of fermentation as proteolytic, lactic acid, thermogenic, will enable the investigator to separate the organisms into their respective groups

TABLE 2

Pressure and Temperature Relations

TEMPERATURE		PRESSURE ABOVE NORMAL	
°C.	°F.	Pounds per square inch	Atmosphere
100	212	0.0	0.0
112	234	7.5	0.5
116	240	10.0	0.75
122	250	15.0	1.00
127	260	20.0	1.25
128	262	22.5	1.50
131	267	25.0	1.75
134	274	30.0	2.00

on the basis of their biological activity as determined in test tube experiments.

Anaerobic conditions can readily be attained by physical exhaustion of the air; absorption of atmospheric oxygen with pyrogall, deep stab cultures, by boiling liquid media, cooling and overlaying with mineral oil, vaseline or paraffin, displacement with nitrogen or oxygen, etc.; symbiotic culturing as with *Staphylococcus aureus*; the addition of fresh animal or vegetable tissues in long columns of suitable broth; the inverted plate method or any combination of such procedure. Anaerobic spores can readily be detected by inoculating sterile litmus milk medium and overlaying with sterile paraffin, followed by heating the tubes in a boiling water (100°C.) bath for ten to twenty minutes and with subsequent incubation.

Culture media should be sterilized at the lowest temperature that will insure sterility in the shortest period of time. Using an autoclave at 15 pounds pressure (above normal atmospheric pressure) for twenty minutes is entirely satisfactory in most cases. Sugar media should preferably be sterilized at 10 pounds pressure for the same period of time. The use of steam under pressure is the most useful process of sterilizing apparatus in bacteriological procedure.

Although many fermentations occurring in nature are produced by mixed flora it is seldom that more than two species are concerned in any particular phase of the process. Such fermentations are often referred to as "predominating type" in which the desired species are added as a starter culture and other similar organisms enhance the desirability of the finished product without having a direct relation to the major product formed.

NOMENCLATURE

The binominal system of nomenclature according to the Swedish naturalist Carl von Linne (Linnaeus) was the first plan of uniformity in the assignment of the names of plants. The various classifications as are now recognized in bacteriological procedure follow closely this general plan of the botanists. The first name is the genus designation or the generic name and the second is the species designation or the specific name. In all microbiological work reference should be made to the causative agent in a biological process as *Bacillus subtilis*, *Clostridium butyricum*, *Saccharomyces cerevisiae*, *Aspergillus niger*, *etc.*

The classification most generally used by American bacteriologists after 1900 was a system by Migula. Chester in 1901 adopted this classification and is largely responsible for its use in this country. In 1905 Orla-Jensen arranged an extensive classification of the bacteria by including biological as well as morphological characteristics. Buchanan in 1915 contributed valuable information with his new classifications. Later in 1919, Castelani and Chalmers gave extensive classification of the tropical pathogens. In 1930 Bergey assisted by a committee of the Society of American Bacteriologists classified the bacteria on the bases of morphological and biological characteristics.

REFERENCES

(1) Hering, C.: U. S. patent 1,355,476, 1920.
(2) Bart, H.: U. S. patent 1,250,079, 1917.
(3) Rubner: Arch. f. Hyg., xi, 1890.

Section I

THE PRODUCTION OF CARBOXYLIC ACIDS

CHAPTER 2

Acetic Acid and Vinegar Production

HISTORICAL

It is noticed that if wine or beer or in fact any alcoholic liquid is left exposed to the air a tough skin or membrane will be found to cover the liquid in a few days. The alcohol in this wine or beer gradually disappears and in the same ratio acetic acid gradually appears. It is also a matter of evidence that any sample of alcohol if inoculated with such a pellicle or skin will be converted into vinegar. This pellicle has always been regarded as the carrier of vinegar fermentation and as such has been known and is known as the "mother of vinegar." Persoon (1), in 1822, named this skin Mycoderma. This investigator, however, did not understand the connection between this skin and vinegar fermentation. Kutzing (2), in 1837, showed that the "mother of vinegar" consisted of bacteria. He classified these organisms as algae and named them *Ulvina aceti*. Liebig (3), two years later, appeared with his new theories regarding fermentation. He asserted that the "mother of vinegar" was a formation entirely devoid of life. Thomson (4), in 1852, showed that the "mother of vinegar" constituted 94.53 per cent organic material and 0.336 per cent ash. Pasteur (5), in 1864, showed that acetic acid fermentation was a physiological process whose inception is bound up with the activity of a minute organism which he named *Mycoderma aceti*.

In natural acetic fermentations the reactions are rarely carried through by a single species of Acetobacter. Hansen (7), as early as 1879 showed that two species might be associated, which he named *Mycoderma aceti* and *Mycoderma pasteurianum*. Since then a number of other pellicle forming and some non-pellicle forming Acetobacters have been described. Bergey (31) lists eleven and Hooft (26) six, some of which do not form pellicles.

These organisms as a class are decidedly pleomorphic. They may exist singly or in chains and have a tendency to form extremely long threads often irregular in shape and with swollen or bulged forms.

More recently Hansen (11), 1894, Beyerinck (14), 1898, and Henneberg (13), 1898 worked with the vinegar bacteria. Janke (18), in 1916, classified the bacteria mainly according to the morphological differences. Bergey (31), classifies the bacteria into the genus Acetobacter, consisting of eleven species. These bacteria, according to the aforementioned men, are described as possessing the power of forming a pellicle on liquid media. The various species have been classified according to the thickness and the quality of this pellicle. Kluyver and De Leeuw (24), in 1925, described a new species, *Acetobacter suboxidans* which formed no pellicle. This species is able to form calcium-oxygluconate on yeast extract agar plus 2 per cent calcium carbonate and 2 per cent glucose. Vandecaveye (27), in 1927, reports the formation of acetic acid in very large quantities from cull apples. The dominating strain in this reaction was a bacterium that never formed a pellicle. In this author's work eight strains of the non-pellicle forming bacteria were used in the study. He suggests two new species in addition to *A. suboxidans* which he says however may be varieties of *A. aceticum* and *A. rancens* of Hooft. They are rapid acetic acid formers.

VINEGAR FROM SUGAR

The fermentation of vinegar from sugar is a process in which there are two individual and distinct steps. First, one organism, namely, yeast, acts upon the sugar and converts it into ethyl alcohol. In this process carbon dioxide is liberated as a by-product.

$$C_6H_{12}O_6 = 2C_2H_5OH + 2CO_2$$

Certain bacteria such as *Acetobacter peroxidans* act upon the ethyl alcohol and convert it into acetic acid, according to the following two equations:

$$2C_2H_5OH + O_2 = 2CH_3CHO + 2H_2O$$
$$2CH_3CHO + O_2 = 2CH_3COOH$$

Small amounts of acetic aldehyde can invariably be detected in this fermentation. It is not due, however, to a secondary process but merely represents a momentary stage in the course of the reaction, in which a production and further oxidation of the aldehyde is continually taking place.

According to Neuberg and Windisch (25) the conversion of alcohol

to acetic acid is not a direct oxidation but through acetaldehyde as an intermediate, all acetobacters being able to form acetic acid from acetaldehyde by a dismutative process. Hydrogen is removed from ethyl alcohol to form acetaldehyde.

$$2C_2H_5OH + O_2 = 2CH_3CHO + 2H_2O$$
$$2CH_3COH + H_2O = CH_3COOH + CH_3CH_2OH$$

Two molecules of acetaldehyde are then oxidized to one of acetic acid and one of alcohol. Part of the alcohol used to form aldehyde is recovered as alcohol and reused.

The alcoholic yeast, *Saccharomyces ellipsoideus*, is very well suited to carry on the alcoholic fermentation in vinegar manufacture. This yeast may be described as follows (16). The cells are ellipsoidal. The spores are 3 to 4 microns in length and between 3.5 to 4 microns in width. Jorgensen (15), describes the species as a bottom fermenting yeast. It ferments levulose, dextrose and saccharose but does not ferment lactose. This species exhibits a peculiar reticulated structure on gelatin at the end of eleven to fourteen days, which distinguishes it from other species.

Certain mineral elements, nitrogenous substances and certain hydrocarbon compounds are essential for the growth of the yeasts. The absence of any one of these classes of compounds may cause a cessation in the life activities of these organisms. Mayer (6), has studied in detail the various chemical elements which are necessary for the growth of the yeasts. He believes that yeasts grow best when mineral salts are present in the following proportions:

(1) 0.1 gram monobasic potassium phosphate per 100 cc. water
(2) 0.1 gram magnesium sulphate per 100 cc. water
(3) 0.1 gram tribasic calcium phosphate per 100 cc. water.

According to this author potassium phosphate is the most important mineral compound after which follows magnesium sulphate. These mineral compounds are present in the same proportions in the yeast cells. These observations of Mayer have been confirmed by Elion (10) and Stern (12). These later authors also maintain that sulphur is indispensable in the life of the yeast.

In the nutrition of the yeasts the various ammonium salts are essential for growth. Pasteur (5) first established this fact which was later confirmed by Duclaux (8) and others. Laurent maintains that the yeasts do not use nitrate nitrogen as food.

The yeasts are not able to take their carbon from the atmosphere. Therefore this class of microörganisms must get their carbon from other compounds such as sugars, aldehydes, acids, etc.

Yeasts live both in the presence and in the absence of oxygen. When living an aerobic existence they assimilate oxygen. Under these conditions the yeasts decompose the sugar medium, using part of it for maintaining metabolism and transforming the rest of it into carbon dioxide and water. In this capacity the yeasts act as typical plants. On the other hand, if the supply of oxygen is cut off, very little sugar is utilized for life activities. In this condition the yeasts scarcely multiply. The bulk of the sugar in the medium is transformed into alcohol and carbon dioxide. Thus, the conditions necessary for alcohol production are anaerobic in character. The energy of the yeast, in this latter instance, is not secured by oxidation but is rather secured by the enzymatic change of the sugar into alcohol and carbon dioxide.

Wyant (20), of the Michigan Agricultural Station says that in the alcoholic fermentation there should result about as much 50 per cent alcohol as there was sugar in the unfermented solution. She says, "100 parts of sugar in the juice should produce theoretically about 51 parts of alcohol." In actual practice only from 43 per cent to 47 per cent alcohol is obtained because some of the sugar is utilized for nutrient purposes.

A variety of organisms may convert the alcohol formed into vinegar. Among these may be mentioned *B. aceti*, *B. pasteurianum*, *B. kuntzingianum*, *B. xylinum*, *B. acetigenous*, *B. oxidans*, and *B. industrius*. In addition two other species, namely, *Acetobacter suboxidans* and *A. peroxidans* may be utilized in acetic acid production.

The conversion of alcohol into acetic acid is an oxidation and as such an abundant supply of oxygen is presupposed. Therefore, the organism causing the oxidation must be present on the surface of the liquid. It has been ascertained that one vinegar bacterium is capable of fermenting hundreds of times its own weight of acetic acid. However, it has also been proven that after 8 per cent acetic acid has been formed the action of the bacterium becomes less and less vigorous and by the time 12 per cent to 14 per cent acetic acid has been reached the bacteria have entirely ceased to act.

Wyant (20) states, ". . . . for every hundred parts of sugar present in the original sugary solution, 50 to 55 parts of vinegar should be

obtained under favorable conditions. So if a vinegar containing 5 per cent acetic acid is desired, the fermentation should be started with at least 10 per cent sugar solution."

It has been ascertained that the acetic acid bacteria will destroy the acetic acid formed if the alcohol solution is allowed to drop below 2 per cent. This phenomenon occurs according to the following equation:

$$CH_3COOH + 2O_2 = 2CO_2 + 2H_2O$$

Thus the acetic acid produced is broken up into carbon dioxide and water.

Acetobacter suboxidans which is a typical member of the non-pellicle forming group is a rod occurring singly and in chains. It measures 1 to 2 microns in length and 0.8 to 1 micron in width. It is Gram positive, non-motile and non-spore forming. On agar slants, the organism is characterized by a spreading, moderate and raised growth. On agar plates *A. suboxidans* grows most rapidly forming circular colonies. No pellicle is formed on bouillon. Litmus milk agar remains unchanged and apparently no growth takes place. Acid is produced from glucose, mannitol, sucrose, lactose, ethyl alcohol, propyl alcohol, butyl alcohol and glycol. This organism has been used with excellent results by students in Smyth's laboratory.

Anything may be used in making vinegar so long as it contains the proper sugar content. Nearly all varieties of apples contain the proper sugar content with which to make vinegar. According to LeFevre (23), of the Bureau of Chemistry the average sugar content of apples is 13 per cent and in no case does the percentage fall below 10 per cent. Summer apples cannot be utilized for vinegar manufacture because the sugar content is too low. Green apples may also not be used because the starch has not yet been converted into sugar.

A very high quality of vinegar may be made from grapes. Two types of grapes may be utilized in the manufacture, namely the whole white grapes and the pulp from red grapes. Grape juice is very high in sugar, therefore a much stronger vinegar results.

Cull oranges yield a very acceptable vinegar. Certain varieties of peaches (Carmen type) may also be used in vinegar manufacture. Certain grains, as for example, corn, barley, rye, and oats have also been used.

Several methods of manufacture may be given for the production of vinegar in the home, the farm and in industry.

HOME MANUFACTURE

Jars are usually utilized for the home manufacture of vinegar. These jars have a capacity of from 3 to 6 gallons. Ripe fruits, having the proper sugar content are selected for the process. The jars are filled two-thirds full. A cake of yeast is mixed with a small amount of the mash which is then mixed with the entire mash. A cheese cloth is then placed over the jars to exclude any insects. This mash is now placed away and the alcohol fermentation is allowed to proceed. The mixture is stirred from day to day to break up the surface crusts and to prevent the formation of molds. When this fermentation is complete the liquids are separated from the mash by straining through cheese cloth. The juice is then again returned to the jar. One may follow one of two methods. One may introduce the acetic acid culture directly or one may introduce a starter of vinegar in the proportion of one part of vinegar to four parts of alcoholic liquid. During the acetic acid fermentation tests are made to determine the acidity of the liquid and when the proper acidity is reached the fermentation is stopped, the vinegar filtered and bottled (23).

FARM PRODUCTION

In making vinegar for the farm, apples are generally used. After pressing the juice is placed in loosely covered barrels for sedimentation. The juice is then siphoned off and is placed away for alcoholic fermentation. Yeast is added to the entire mixture in the proportion of 1 cake of yeast to every 5 gallons of juice. As in the previous case the yeast is first mixed with a small part of the juice which is in turn mixed with the entire batch.

There are several methods of carrying out the acetic acid fermentation. Among these may be mentioned the Slow Barrel Process (23), the Continuous Process (23), and the Rolling Generator Process (23).

The Slow Barrel Process may be described as follows: The alcoholic juice is transferred to barrels lying on their sides. Numerous bung holes are bored in the sides of the barrels to insure an abundant supply of oxygen. About 3 gallons of unpasteurized vinegar are then added to each barrel. Then the acetic acid fermentation is allowed to proceed. When the process nears completion numerous tests are made in regard to the acidity of the resulting acid.

One recognizable factor is noticed in all three processes and that is the free circulation of air. It may also be noticed that in the farm

methods of manufacture the time necessary for complete acetification is long, three to six months being required for the entire process.

COMMERCIAL ACETIC ACID PRODUCTION

Commercially, the formation of acetic acid is hastened by allowing the alcoholic mixture to trickle through a tall wooden vat containing wood shavings which have previously been inoculated with acetic acid bacteria and through which a flow of air is continually passing. It is noticed that the process of acetic acid fermentation is materially hastened because of the great exposure of the alcoholic liquid to the air and to the acetic acid bacteria. Such a process is known as the Quick or Generator Process of Making Vinegar. By this method vinegar may be manufactured in a few days that may take months in the processes otherwise enumerated.

While pure culture fermentation of sterilized material is not the rule in vinegar manufacture yet it is advisable to ensure maximum production and controlled flavor. Usually, as previously stated, acid fermentation of the alcoholic liquor is started by the addition of 10 per cent of unpasteurized vinegar or "a starter may be prepared by exposing a suitable liquid in a shallow vessel to the air of a warm room for several days" (17). This liquid may be a fermented fruit juice or a yeast extract made by boiling 50 grams of fresh yeast with a liter of water to which is added a small amount of vinegar or wine.

This starter in a few days develops a film of acetobacters. The film may be floated on the surface of the liquor to be seeded by means of a spoon or spatula or chips of beechwood may be used as rafts to float the "skin."

A better practice, not usually followed, would be to isolate one or more acetobacters in pure culture and use these as starters. Where non-pellicle forming organisms such as *A. suboxidans* are used no floating will be needed. Details of vinegar production methods are available in pamphlet form in bulletins issued by the United States Department of Agriculture (23), the Iowa Agricultural Experimental Station (21) or University of California Experimental Station. An excellent dissertation on vinegar production is to be found in Creuss's *Commercial Fruit and Vegetable Products* (22).

In small batches of fermenting solutions a rough estimate of the rate of alcohol formation may be made by loss in weight due to evolution of CO_2. When the fermenting solution ceases to lose weight fermenta-

tion has ceased. At the end of the yeast fermentation the alcohol yield may be determined by distillation of an aliquot portion, dilution of the distillate to the volume of the original and determination of the specific gravity by the Westphal balance according to the official methods of the Association of Official Agricultural Chemists (29).

The rate and completeness of fermentation may also be followed by determination from time to time of the residual sugar by the Munson and Walker method (29).

Acetic acid formed is estimated by titration of an aliquot with N/10 NaOH solution, 1 cc. of N/10 NaOH is equivalent to 0.006 gram acetic acid. Yields may be calculated on the basis of reducing sugar as dextrose in the original must and as percentage conversion of alcohol formed to acetic acid.

Acetobacters may be isolated from vinegar or fruit juices by the serial dilution plate method using nutrient agar containing 10 per cent of fresh cider.

The fermentation of distillers' wash and other materials to produce acetic acid and other organic acids is described in a patent issued in February 1930 (30). The wash from the still after removal of alcohol is sterilized, adjusted to a pH value of 7 and fermented with an organism designated as belonging to the *Bacillus aminophylus* species. After fermentation the volatile acids are distilled off and the residue concentrated and used for fertilizer. This species name does not appear in Bergey's *Manual* but possibly it is similar to or related to *Bacillus aminovorans*.

ACETOBACTERS

As many of the acetobacters might be used to produce vinegar or acetic acid we give a synopsis of the tribe, taken from Bergey, including carbohydrates acted upon, optimum temperature and type of pellicle where described. Hooft's classification is also given.

Acetobacter: Cells rod shaped, frequently in chains. Motile or non motile. Usually grow on the surface of alcoholic solutions as aerobes, securing growth energy by the oxidation of alcohol to acetic acid. Also capable of utilizing certain other carbonaceous compounds, as sugar and acetic acid. Elongated, filamentous, swollen and even branched forms may occur.

A. Motility generally absent.

a. Form acetic acid in dextrose, ethyl and propyl alcohol and glycol.

1. *A. pasteurianus.* Opt. temp. 30°C. Dry, wrinkled, folded pellicle of fluid media.

2. *A. acetus.* Opt. temp. 30°C. Slimy pellicle on fluid media.
3. *A. kutzinganus.* Opt. temp. 30°C. Rather thick folded pellicle.
4. *A. lindneri.* Opt. temp. 25°C. Dirty yellow brown pellicle.

aa. Form acetic acid in dextrose, galactose, ethyl and propyl alcohol and glycol.

5. *A. acetosis.* Opt. temp. 30° to 36°C. No pellicle described.

aaa. Form acetic acid in dextrose, galactose, ethyl and propyl alcohol, glycol and sucrose.

6. *A. xylinus.* Opt. temp. 30°C. Forms a film on beer which becomes cartilagenous and falls to the bottom.

aaaa. Form acetic acid in alcohol.

7. *A. ascendens.* Opt. temp. 25°C. Forms a tough pellicle rising on the walls of the flask.
8. *A. plicatus.* Opt. temp. 25° to 30°C. Thick pellicle.

AA. Motile.

a. Form acetic acid in dextrose, ethyl and propyl alcohol and glycol.

9. *A. acetigenous.* Opt. temp. 30°C. Tough slimy pellicle.

aa. Form acetic acid in dextrose, ethyl and propyl alcohol, glycol, arabinose, levulose, maltose, lactose, raffinose, dextrin, glycerol and manitol.

10. *A. oxydans.* Opt. temp. 18° to 21°C. No pellicle described.
11. *A. industrius.* Opt. temp. 25°C. Forms pellicle.

Visser T. Hooft Classification of Acetobacters based on oxidation reactions

A. Catalase negative.
  I. Oxidize gaseous H_2
    1. *Acetobacter peroxydans*
  II. Not oxidize gaseous H_2
B. Catalase positive
  I. Grows in Hoyer's solution
    2. *Acetobacter aceticum*
  II. No growth in Hoyer's solution
    a. Form acids and carbonates on alcohol calcium carbonate agar
      3. *Acetobacter rancens*
    b. Form only acids on alcohol calcium carbonate agar
      bb. Form gluconic acid from glucose calcium carbonate agar plates and later oxidize to calcium carbonate
        4. *Acetobacter xylinum*
        5. *Acetobacter melanogenum*
      bbb. Form gluconic acid from glucose calcium carbonate plates and later oxidize to calcium oxy gluconate
        6. *Acetobacter suboxydans*

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CHAPTER 3

Butyric Acid and Aromatic Esters

MIXED CULTURE FERMENTATIONS

It has been recognized for many years that there were a number of spore forming soil bacteria that would decompose organic matter by gassy fermentation with the formation of butyric and other fatty acids. The proportion of the various end products in these fermentations depends upon various factors such as, species and strain of organism used, substrate acted upon, temperature, air supply, etc. These fermentations occur with mixed cultures as well as with pure cultures and the organisms are so resistant that ordinary contaminations will not prevent their growth and enzyme activity. In most of the pure culture fermentations used in industry these organisms would be considered contaminants and, except in the extreme acid conditions of citric acid production, pressure sterilization is essential to exclude them. In butyl alcohol fermentation certain members of this group are used in pure culture but here conditions must be accurately controlled to prevent the accumulation of these fatty acids, though they do occur as intermediates.

In spite of the great variations in yields from mixed culture fermentations there have been a number of patents granted in this and other countries for the production of butyric and other fatty acids and their derivatives from the fermentation of vegetable and animal refuse matter of various kinds by cultures of organisms naturally present in the same, or isolated in mixed culture or non-specific pure culture from similar materials. The one requirement in all these patents is that the organism to be employed be more or less heat resistant.

The first of this group of patents was granted in 1910 to J. Effront (1) for a "Process of Fermenting Organic Nitrogenous Substances" to obtain ammonia and volatile fatty acids. This is the only patent we have found for the production of ammonia by biological activity, though ammonia is an end product in the decay of all nitrogenous organic matter, and ammonification is an essential step in the nitrogen cycle in nature.

The patent states that yeasts and molds always contain germs of butyric ferments which produce and amidase capable of splitting amino-acids. The bacteria are obtained from molds or commercial yeasts by adding either or both to sterilized washes of molasses rendered alkaline and held at 90°C. for a half hour to kill non-spore-formers and then incubated at 40°C. for twenty-four hours to develop a strong fermentation with the evolution of hydrogen gas. (No mention is made here of the CO_2 also produced.) Molasses washes or "spent beers" from alcohol fermentation are preferred, though washes from corn and beets, distillery wastes, sugar factory washes or even wool scouring wastes may be used. Molasses washes give best results when they have a specific gravity of 1.050 to 1.060. This should average 70 to 90 grams of nitrogenous matter and 2 to 3 grams of sugar per liter. If weaker it may be concentrated by evaporation or if stronger first fermentations may be diluted and the organisms gradually adapted by successive subculturing in gradually increasing strength of washes.

Molasses washes should be rendered alkaline with potassium carbonate 4 to 8 grams per liter, after being neutralized with lime if necessary, and reinforced by the addition of 0.06 to 0.1 per cent aluminium sulphate, 0.02 to 0.05 per cent calcium super-phosphate and inoculated with 0.01 per cent pressed yeast and 0.01 per cent mold mycelium. After forty-eight hours active fermentation first batches are added to 3 to 5 volumes of unfermented fortified wash. During fermentations of these seed cultures they are kept agitated by blowing air through. Successive subcultures by the above method are made to obtain sufficient volume of active seed to allow the addition of say 5 to 10 hektoliters to 100 hektoliters of wash to be used for production. These larger volumes are aerated only during the few hours necessary for tank filling as continuous aeration would carry off ammonia as formed.

Any of the materials suggested above are used for production, the patent stating that they all may be rich in amino-acids. Complete conversion is claimed and two illustrative formulae are given.

$$\underset{\text{Glycocoll}}{CH_2NH_2CO_2H} + H_2O = NH_3 + \underset{\text{Acetic acid}}{CH_3COOH} + O$$

$$CO_2HCHNH_2CH_2CO_2H + H_2O = NH_3 + \underset{\text{Propionic acid}}{CH_3CH_2COOH} + CO_2 + O$$

These formulae do not account for the hydrogen gas which the patent claims is formed.

The main fermentations are allowed to continue two to three days or until a sample to which magnesium oxide is added and then distilled gives 90 per cent of the nitrogen as free ammonia. Successive fermentations may be conducted by seeding unsterilized wash, but new seed cultures should be started every fifteen days. Ammonia yields should range from 7 to 7.5 grams per liter. It is recovered by distillation after adding 2 to 3 grams of alkali per liter to the fermented batch. Fatty acid yields should be 70 to 80 grams per liter of combined butyric, acetic and propionic acids. The patent gives directions for their recovery by concentration, liberation with sulphuric acid and fractional distillation. Sewage may be so fermented after neutralization, as may turf after hydrolyzing with sulphuric acid and neutralization.

It is interesting to realize that such a crude process of fermentation of indiscriminate raw materials by undetermined bacteria to yield variable amounts of end products could be patented. In reality the patent office has granted the patentee the right to attempt to control one stage in the nitrogen cycle of nature by the use of an undetermined agency.

In 1914 Le Franc was granted an English patent (2) for the "Manufacture of Acids of the Fatty Series and of Butyric Acid in Particular," using a mixed culture of *Bacillus amylozyme* of Perdix to act on amylaceous substances.

As raw carbohydrate materials he suggests sugar refinery residues, beets, molasses, potato pulp and wood pulp (hydrolyzed). To these materials various nitrogenous substances as fibrin, casein, leucine, knacker's waste, powdered flesh or fish guano, gluten, albumens, etc., may be added.

This conglomerate mass (or mess) is sterilized at 100°C., to it is added an excess of calcium carbonate or magnesium oxide and it is then fermented at 38° to 39°C. On completion of fermentation the mass is filtered, the filtrate concentrated in multiple effect apparatus until calcium butyrate (or magnesium butyrate) begins to crystallize out from the hot liquid. The crystals are filtered out and decomposed with a mineral acid to obtain acetates, propionic, valeric, butyric, caproic, etc., acids. In brief a compost pile of indiscriminate junk has been patented.

In 1918 a patent was granted to Hibbert for a "Solvent and Process of Producing Same" (3). Carbohydrates are fermented to produce butyrates and other salts with butyric ferments, either directly, or indirectly by first forming lactates and the like, and thereafter converting these into salts of acids of the fatty acid series by a subsequent fermentation. The acids are liberated from the salts produced and converted into mixtures containing ketones by treatment with a suitable catalyst at a high temperature. The ketones are either marketed directly or reduced in whole or in part to the corresponding carbinols by hydrogen gas and another catalyst. These carbinols may be acteylated or not as desired.

It is claimed that the extremely variable pleasant smelling liquid is an excellent solvent for cellulose derivatives. Its variable nature is indicated by the boiling range as given 110° to 160°C., the major part from 120° to 145°C.

As raw material any cheap "slop" may be used, as final molasses, whey, yeast slop or distillery slop. To this carbohydrate liquor is added an excess of calcium carbonate and it is then inoculated with a lactic acid organism, type not stated, and allowed to ferment at 35° to 40°C. to form lactic acid and calcium lactate. If yeast slop or whey is used it may not be necessary to inoculate as they may contain sufficient lactic acid formers.

The fermented liquor is then seeded with a butyric ferment, preferably not in pure culture, and further fermented in covered vessels to form fatty acids. The final fermented mass is filtered and evaporated down to crystallization. Further chemical treatment is given in detail in the patent. As alternative the original "slop" may be simultaneously inoculated with both a lactic acid former and a butyric culture. No mention is made as to how the cultures are obtained or from what source and nothing is said about sterilization of liquors to be fermented.

As given in the claim this method would give extremely variable yields, never very high, and its commercial value would be very doubtful. Again we have the patenting of natural fermentation processes by unknown and undetermined organisms with abundant opportunities for failure or poor yields, and with the production of variable amounts of an uncontrolled mixture of decomposition products.

In 1919 two similar British patents were granted to Darasse Freres (4) one for "Treating Seaweeed' or marine algae by fermentation with

cultures found on the same after neutralization with oxides, carbonates or other basic salts of alkalies, alkaline earths or zinc or iron groups.

After fermentation the cellulose residue is acidified and distilled to obtain formic, acetic and butyric acids. The iodine and potassium salts are separated from the residue by chemical means.

The second patent is for "Treating Waste Vegetable Substances" (5) by the same process with fermentation of the carbohydrates contained in plants, vegetable offal, waste products as alkaline or bisulphite lyes from the preparation of paper pulp, sawdust, tannin, corozo waste, grass, leaves, etc. This is another compost heap patented. The United States patent (6) granted to L. Dupont for a "Process for the Utilization of Marine Algae for the Manufacture of Acetic and Butyric Acids" is the same process as the first Darasse patent refered to above.

In 1921 a United States patent was granted A. A. Backhaus (7) for a "Method for the Production of Esters" from carbohydrates by fermentation to acids and the continuous esterification of the higher acids of the paraffin series.

As a raw material any carbohydrates may be used but distillery waste, especially alcohol molasses is preferred. This material is evaporated to a density of 10° Baumé or higher, and then inoculated with butyric bacteria (type not specified) obtained from any source, such as swamp mud, cattle feces, garden soil, ensilage, saurkraut, decaying grapes, fruit, etc. Sterilization of substrata is not indicated. The inoculated mass is held at 35°C. for several days and then fed into an esterification apparatus, described and illustrated in the patent, where it is acted upon by sulphuric acid and methenol or other acids and alcohols as desired. Wastes are normally acid but they may be rendered neutral or alkaline with calcium salts if desired. Lower temperature fermentation favors acetic acid production and higher temperatures butyric. In a sample run quoted for 40 parts by weight of acetic acid there were formed 45 parts each of propionic and butyric acids.

In 1922 McDermott and Glascow (8) were granted a patent for the "Manufacture of Butyric Acid" from waste liquors from distilleries, breweries, yeasts and vinegar factories, spoken of as "spent beer" or "slop."

These slops contain small amounts of glycerol, pentoses, pentosans,

proteins and simpler nitrogenous bodies, mineral salts, lactic and succinic acid.

While still hot (90°C.) they are almost neutralized with anhydrous lime and then an excess of calcium carbonate is added (or it may be added periodically during fermentation).

This neutralized slop is inoculated with a culture from malt sprouts or hay which may contain *Bacillus subtilis*, *Granulobacter saccharobutylicum* or *Bacillus boocopricus*. The inoculation is made into the hot mass to kill off contaminating non-spore forms, but no further attempt is made to obtain a pure culture.

After a short time (presumably thirty minutes or so) the temperature is lowered (point not stated), and fermentation is conducted in closed kettles with the production of butyric acid or calcium butyrate. No mention is made of other fatty acids which must be formed. The acid is recovered with sulphuric acid and distillation in the usual way.

Le Franc was granted United States patent, in 1927 (10), for the "Manufacture of Butyric Acids and Other Aliphatic Acids," similar to his British patent (2) but using as raw material waste wood powdered and hydrolyzed with 2 per cent sulphuric acid at 170°C. for thirty minutes, then discharged into an enameled mixer at atmospheric pressure. (Similar to the principle employed in making puffed rice, and to the process of making Masonite fiber board.) It is then mixed with chalk in excess, if necessary concentrated to 8 to 12 per cent of wood sugar (presumably calculated as dextrose). If very turbid it may be clarified with milk of lime.

It is then fermented in closed vats at 40°C. after inoculation with cultures obtained from the feces of herbivorous animals or from garden soil. They are isolated in sugary solutions, glucose or saccharose, and transferred for four or five generations at 40°C. No further attempt is made to obtain pure cultures. Before fermentation there is added 5 to 7 per cent of powdered calcium carbonate and 0.5 per cent each of potassium phosphate and ammonium nitrate or sulphate.

Fermentation is continued for from six to fifteen days or until gassing ceases. The slop is then evaporated to extract the calcium butyrates, propionates, acetates, caproates and valerates, the acids being recovered in the usual way.

In 1928, a French patent was granted to Le Ketol (11) for butyric acid production by direct fermentation of amylaceous substances

without previous saccharification. A wort of starch or starchy material is prepared, a nutrient is added, as sugar factory waste, and also inorganic salts. The mass is fermented with a pure culture or with symbiotic butyric cultures.

Allied to this group of butyric fermentation patents, but differing in that other substances are also recovered, in a United States patent granted, in 1926, to W. M. Sinclair (9) for a "Process of Manufacture of Aldehydes, Oils and Organic Acids from Cacti." Here the raw material is the prickly pear plant, which is an ideal fermentable medium, no watery addition being needed. The cacti are crushed on rolls and filled into closed vats equipped with stirers and held at 20° to 35°C., with agitation once or twice daily, for about five days.

No seeding with cultures is necessary as there is present on the cacti a symbiotic fungus indispensable to its growth. These organisms are not described or named, but they are claimed to convert soil humus and dead cactus tissue to suitable food for the living plant. Apparently they function as do the radicicola for leguminous plants. The patent states that the pear protoplasm is usually very rich in mesoxalic acid.

Vats for fermentation may be glass or glass-lined metal, or of wood free of essential oil. No zinc or copper vessels should be used. At the end of fermentation the aldehyde is present in the pulpy mass as such, or in a polymeric modification, or as a base and associated with the essential oil of the pear.

Sulphuric acid to 1 per cent of the pulp volume is added and the mass distilled with a slowly rising temperature up to 125°C. The condensate (15°C.) will be a mixture of aldehyde, acid, water and oil, the oil being separated by decantation.

Chemical methods of separation of the aldehydes and acids, largely tartaric and gallic, are given. The aldehyde may be converted to alcohol by reduction in a stream of hydrogen gas over nickel powder.

This group of patents is given in considerable detail, not because the processes are thought to be particularly useful, but as an illustration of the vague indefinite processes that may be or have been patented. There is a very great similarity between them. All ferment rubbish, in most instances of a rather indefinite nature, either animal, vegetable or mixed.

They take advantage of what are considered deleterious influences in the butyl alcohol fermentations, the presence of lactic acid forming

contaminants and the influence of high temperature. The predominating fermentation is the same as the first stage of butyl alcohol production with its high acid values, a good butyric acid fermentation being the equivalent of a badly contaminated butyl alcohol fermentation.

They do utilize waste materials not suitable in composition or concentration for pure culture and definite yield fermentations. Starting with dilute slops of varying composition and fermenting with variable mixed cultures, yields must of necessity be very variable in quantity and in the relative preponderance of the various acids formed. They would be impractical as a method of fermenting pure or homogeneous raw materials with any market value, but they may serve as by-product processes to handle wastes from other industries that would otherwise have no market value.

No detailed scientific studies of these processes are quoted. In fact the biological phases of the processes are unscientific. There is no practical difference biologically between the different patents in this group, except possibly the last mentioned, and the only excuse for the granting of so many overlapping patents is the differences in chemical treatment of the products of fermentation.

Six of the patents stop with acid production and in principle are identical and in operation very similar.

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CHAPTER 4

Citric and Oxalic Acid Production

CITRIC ACID MARKET

A short article in *Food Industries* (59) for August, 1929, refers to the shortage of citric acid in England with a tendency in Sicily to improve the quality of the lemon output. This is diverting considerable of the present raw material for citric acid production by changing low grade material now used for that purpose to higher grade fruit suitable for export to meet the steadily increasing demand for lemons. Great Britain does not produce citric acid by synthetic or mycoidal processes, imported calcium citrate being their sole source of supply. These facts are taken by the authors to point to an increasing opportunity for American manufacturers of citric acid by biological or synthetic processes.

HISTORICAL—OXALIC ACID

It has long been recognized that quite a large group of molds will ferment carbohydrates to produce oxalic, citric or other organic acids. Oxalic acid production by fungi was first discussed in considerable detail by Wehmer in 1891 (1) in a series of twenty-three short articles in the *Chemiker Zeitung*. He studied this reaction in many species of fungi, especially Penicillia, Aspergilli and Mucors. Oxalic acid has never been produced commercially by this method, however, as it cannot compete economically with pure chemical methods.

CITRIC ACID

In 1892 (2) he described a particular group of Penicillum-like fungi, a number of which, in addition to or at times almost to the exclusion of oxalic acid, would form large amounts of citric acid when grown on carbohydrates in the presence of suitable nitrogenous matter and inorganic salts. These fungi he named Citromyces, *Citromyces pfefferianus* and *C. glaber* being the ones with which he obtained best results. These fungi form a green felt 0.5 mm. or more thick on sugar solutions and will ferment up to 50 per cent of glucose with the pro-

duction of an acidity of up to 8 per cent. From 11 kgm. of glucose he obtained 6 kgm. of citric acid (3). He has isolated 13 more species which he classified as citromyces (4).

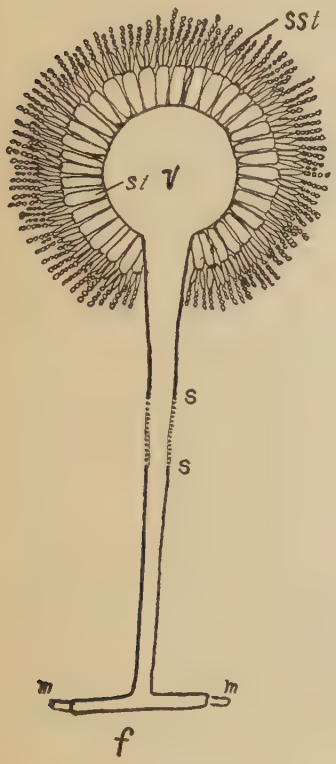


Fig. 1

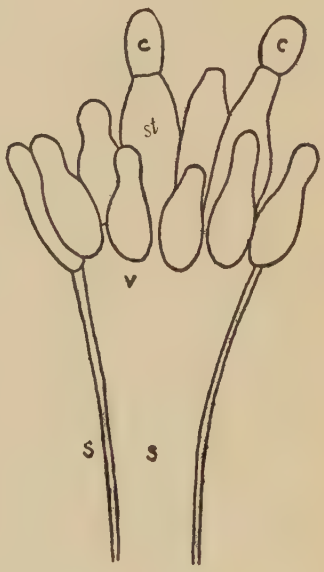


Fig. 2

Fig. 1. Diagram of a Stalk and Head of Aspergillus

f, the foot cell; *m*, vegetative cells at each end of the foot cells; *s*, stalk interrupted since it would be ordinarily much longer; *v*, the vesicle; *st*, primary sterigmata; *sst*, secondary sterigmata with chains or radiating conidia. (After Thom.)

Fig. 2. Aspergillus Head with Simple Sterigmata

s, stalk; *v*, vesicular area; *st*, sterigmata; *c*, developing conidium. (After Thom.)

PATENTS

In 1894 he took out patents in Germany and in the United States (5, 6) for a "Process of Making Citric Acid" by the aid of the above mentioned organisms. He used glucose or other carbohydrates in 10 to 20 per cent solutions with the addition of ammonium nitrate, dipotassium phosphate and magnesium sulphate as nutrients and calcium carbonate to neutralize the acid formed.

In 1913 a United States patent was granted to Beleslas Zahorski (26) for the production of citric acid from carbohydrates by fermentation with a fungus termed *Sterigmatocystis niger*, increasing the acid tolerance of the fungus by growing stock cultures in increasing concentrations of citric acid. Citric acid was also added to the sugar solution to be fermented.

Two United States patents for citric acid production were granted in 1928, the first to J. Szucs (57) for production from molasses, using species of Citromyces, Mucor, Aspergilli or Penicillia selected on the basis of maximum yield. Fermentation was conducted in flat open pans, the acid to be neutralized as formed, or on completion of fermentation, with solutions of alkalies or alkaline earths, preferably barium oxide, carbonate or hydroxide.

The second citric acid patent in 1928 was to Fernbach and Yuill (58) and employed dark colored Aspergilli selected on the basis of yields. Their acid tolerance was increased by growth on sugar media acidulated with mineral acid. Fermentations were to be conducted with a sugar solution adjusted to a pH value of 1.2 to 1.5 with hydrochloric acid. Yields were stated to be up to 65 per cent of the sugar used and the patent called for the production of alcohol from the unused sugar by yeast fermentation.

EXPERIMENTAL WORK

Since Wehmer's first publications this fermentation has been studied in detail by many workers, most of them employing Wehmer's Citromyces or *Aspergillus niger*. The latter seems to be the organism of choice though most workers point out that there is much variation in the amount and type of acid produced by different strains of the same species. It must also be borne in mind that the term *Aspergillus niger* is used to designate "a whole group of black Aspergilli with fundamental characters in common" (53). The fungi considered in this and other fermentations are described in a separate chapter.

GROWTH AND METABOLISM OF FUNGI

Nikitinsky (10) found that alterations in culture fluid of fungi may produce changes in their metabolism which will persist through some generations after transfer to other fluids. Acid tolerance may be included here but will be discussed in detail later. Pringsheim (16) studied the influence of amount and concentration of nutrient on fungus growth. He found that in nutrient solutions of similar composition the amount of mycelium formed depends upon the volume of the fluid. In different concentrations of solutions the concentration influences the rapidity of growth, up to a definite maximum limit. Small amounts of toxic substances may act as stimulants to growth.

EFFECT OF METALS ON GROWTH

Richards in 1896 (8), studying the effect of various chemicals on fungus growth, found that aluminum had no action, but silica in concentrations of 0.3 to 0.5 per cent was slightly stimulating. The following elements proved to be stimulating to growth in the mentioned concentrations.

Cobalt	0.002 per cent
Nickel	0.033 per cent
Manganese	0.066 per cent
Zinc	0.0005 to 0.008 per cent with 0.002 to 0.004 optimal
Iron sulphate	0.033 to 0.2 per cent

He stated that minimal amounts of zinc were stimulating though not essential to growth, but that small amounts of iron were essential. This latter fact was disproved later by Currie (29) although he and Niethammer (51) confirmed its stimulating effect. Lappaleinen (31) states that even the small amount of zinc that may be present in glassware employed will stimulate growth of mycelium but may lessen conidia formation. Wachter (13) tested the toxic action of various chemicals on *Aspergillus niger* but his findings do not seem to have direct bearing on this process. Lafar also summarizes the results obtained by various workers as to the effect of various elements on Aspergillus metabolism (22).

Currie (29) found that the addition of zinc or other stimulants increases mycelium volume but not acid yields. Iron was not essential.

NITROGEN

Butkewitsch who has written at length on the activities of these fungi studied the action of *Aspergillus niger* on non-sugar media with peptone or fibrin as the sole organic source of nutrient. On such media it forms chiefly ammonia as the nitrogenous end product, while *Penicillium glaucum* and the Mucors form chiefly tyrosin and leucin. In the latter case the medium becomes alkaline due to ammonium carbonate formation, while in the former the formation of free oxalic acid keeps the reaction acid and results in the further degradation to ammonia. If the Penicillum culture is acidified with phosphoric acid then ammonia will predominate as with Aspergilli. The proteolytic enzymes responsible for these actions are both intra- and extra-cellular in character. In no case is much ammonia formed if sugars are present.

Lafar (22) discussing conidia formation by *Aspergillus niger* says that Tauret found no conidia were produced on Raulin's solution (stated by Thom (53) to be the first synthetic culture medium used for fungi), containing 0.5 per cent or more of ammonium nitrate or larger quantities of the sulphate or chloride, when grown at 20° to 22°C. Ammonium nitrate retards but does not prevent conidia formation. Free acid is liberated from the salts. Ammonium phosphate up to 2 per cent stimulates growth. When air is excluded from *Aspergillus niger* cultures they soon cease to give off carbon dioxide and they will die within one hour, even on sugar solutions.

ENZYMES

Lafar states that Aspergilli produce nearly all the enzymes known. Herzog and Polotzky were unable to recover enzymes from killed cultures (20, 21). They were unable to detect any citric acid formation from the action of killed cultures on dextrose but later were able to produce citric acid from glycerin with killed cultures. Muller in 1926 (54) reported the production of a gluconoxydase enzyme produced by growth of *Aspergillus niger* on a glucose substrate and obtained from a sugar-free dry preparation of mycelium. This enzyme formed acid and carbon dioxide from glucose but had no action on galactose and fructose. We will discuss the production of tannase, protease and amylase by Aspergilli in other chapters.

ACID FORMATION

Mazé and Perrier (11, 12) in studying the mechanism of citric acid production concluded that it was a product of incomplete respiratory metabolism. Their theories were later condemned by Bernhauer (48). Lafar gives simple chemical formulae expressing the formation of citric and oxalic acids from sugars by mold action but it must be realized that these formulae merely indicate substrate and end-products and take no consideration of the complicated and not as yet fully explained intermediate steps:

$$C_6H_{12}O_6 + 3O = C_6H_8O_7 + 2\ H_2O$$

Citric acid

$$C_6H_{12}O_6 + 9O = 3\ C_2H_2O_4 + 3\ H_2O.$$

Oxalic acid

In the presence of calcium carbonate the citric acid forms calcium citrate from which it may be recovered by quanitative sulphuric acid addition:

$$2\ C_6H_8O_7 + 3\ CaCO_3 = Ca_3(C_6H_5O_7)_2 + 3\ H_2O + 3\ CO_2$$

$$Ca_3(C_6H_5O_7)_2 + 3\ H_2SO_4 = 2\ C_6H_8O_7 + 3\ CaSO_4$$

ACIDS

Buchner and Wustenfeld (23) discuss citric acid production from Citromyces as carried out commercially in the Fabriques de Products Chimiques at Thann and Muhlhausen in Alsace. They state that citric acid formation cannot be a simple oxidation, due to the differences in structure of the glucose and citric acid molecules, but must be a combined splitting and synthesis. They state that in heavy mold felts only the surface mycelia live and are active in acid production. In media rich in nitrogen mycelial growth is excessive with heavy felt formation but little acid production, while in nitrogen-poor media hypha with low nitrogen content are formed which are especially active to form acids. The nitrogen content of the nutrient solutions falls rapidly in the first weeks to a permanent low level and citric acid formation starts with the lowering of nitrogen.

CARBOHYDRATES ACTED UPON

The various patent claims suggest as suitable raw material: starch, saccharose, glucose, levulose, lactose, invert sugar, maltose, glycerol, molasses and, in broader terms, sugar and sugar-like substances, but cane sugar, glucose and molasses seem to be the most generally used. Wehmer (24) found that glycerin was an excellent medium for citric acid production, using it in from 3 to 20 per cent solutions with the addition of mineral salts. He argues from this that citric acid formation must be a synthesis as it has double the number of carbon atoms as does the glycerin. Working with other carbohydrates he obtained the following yields:

	per cent
Mannite	3 to 6
Xylose, Arabinose, Lactose	5
Sucrose	15
Alcohol	3 to 10

PRODUCTION STUDIES

Currie (29) reports a very interesting series of studies on citric acid production with *Aspergillis niger*, using 50 cc. amounts of sugar medium in 200 cc. Erlenmeyer flasks fermented at 28°C., and larger amounts in shallow pans. He discusses three fundamental factors, the inorganic salt requirement, the general equation of metabolism, and the reaction of the medium. He also states that the chemical reactions resulting in acid formation occur in the mycelium. As the mat grows excessive wrinkling occurs, thus enormously increasing the surface for contact. For quantitative testing of acid production and sugar utilization at the end of fermentation the mycelial mat was drained and washed and the wash water added to the batch which was made up to standard volume by further dilution. The carbon dioxide produced was absorbed in potassium hydroxide, the oxalic acid recovered directly by evaporation and crystallization and the citric acid as the calcium salt. He considers the reaction a three stage oxidation process with successive formation of citric acid, oxalic acid, carbon dioxide and new mycelium. By varying conditions he could vary acid production by the same strain from zero to 50 per cent sugar conversion.

Favorable factors for acid production are low nitrogen, largely as ammonium salts, and high sugar. The use of calcium carbonate was unnecessary as his fermentations would continue to an acidity of 10 per cent or over. He found mineral acids less toxic than organic

acids at the same pH values. Fermentation was more rapid in an acid medium and he recommends adjusting the pH value before inoculation to about 3.4 to 3.5 with fifth normal hydrochloric acid. He found the following to be the best simple medium:

	grams
Saccharose	125 to 150
Ammonium carbonate	2 to 2.5
Potassium dihydrogen phosphate	0.75 to 1.0
Magnesium sulphate	0.2 to 0.25
Water	1000 cc., pH 3.4–3.5

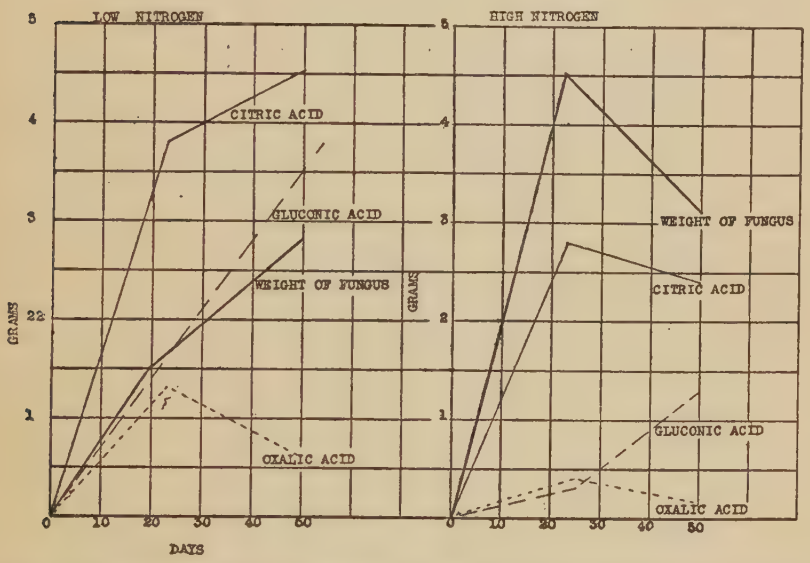


FIG. 3. CURVES SHOWING PRODUCTION OF CITRIC, GLUCONIC AND OXALIC ACIDS ON MEDIA OF DIFFERENT NITROGEN CONTENTS
(After Butkewitsch)

Acid production rises rapidly after two or three days when a good felt is formed and has reached 2 per cent in the next twenty-four hours. Acid increases for seven or eight days and then begins to decline. With good acid yields no spores are formed and the mycelium remains white. See figure 3.

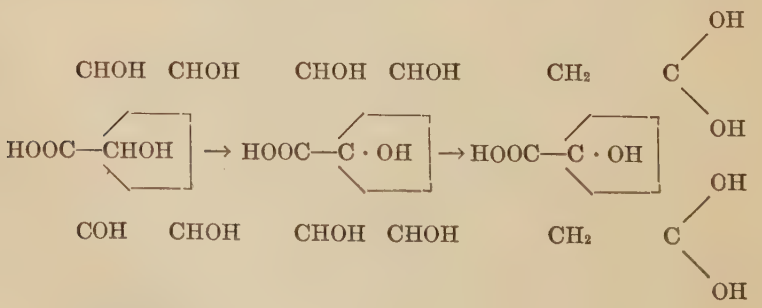
The Fernbach patent previously referred to carries further his suggestion of starting with acid conditions. He makes his mash pH value 1.8 with hydrochloric acid and, in addition to speeding up citric acid production, he is able under these acid conditions to get satisfactory sterilization by a half hour exposure at 100°C. The high acid-

ity also much lessens the danger of bacterial contaminations as no bacteria and few yeasts will grow under those conditions.

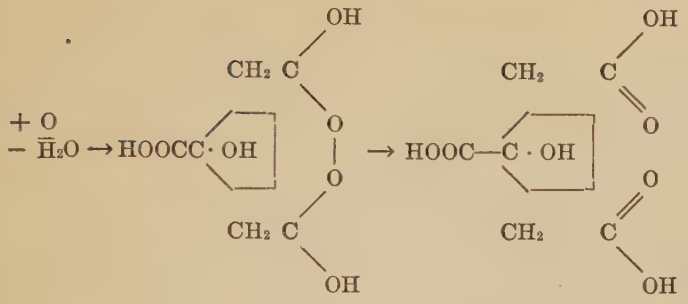
Elfving (32) noted that *A. niger* would not develop on pure solutions of glucose or sucrose but that if a sheet or mat of fungus is grown on a sugar medium containing suitable inorganic salt it will then form acids from pure sugar solutions in the presence of a free air supply. The power of acid formation varies with race. Some strains produce only citric acid and others large amounts of oxalic acid. The presence of calcium salts hinders oxalic acid production. He does not believe that the oxalic acid is formed from citric acid nor that it is a product of intramolecular respiration as Maze believes citric acid is.

GLUCONIC ACID

In 1922 Molliard (33) demonstrated the presence of a third organic acid in *Sterigmatocystis nigra* fermentations which he identified as gluconic acid. About the same time or a little later Butkewitsch (39) also recognized the presence of a third acid in *Aspergillus niger* cultures. He found this fungus to produce citric acid more rapidly than did Wehmer's *Citromyces glaber*. The accompanying graphs from Butkewitsch show the relative amounts of mycelium formation and acid production in media rich or poor in nitrogen. In other papers he (34–41) discussed the formation of citric acid, stating that it is formed directly from sugar with, as a probable intermediate, an acid similiar to parasaccharic acid, which is precipitated by lead acetate. Gluconic acid is not an intermediate in citric acid formation nor is the fermentation related to alcoholic fermentation, there being no acetaldehyde intermediate. Gluconic acid formation begins before that of citric acid. With low acidity gluconic acid predominates and with high acidity citric acid. He suggests a series of graphic formulae for citric acid formation starting with glycuronic acid as an intermediate.



Glycuronic acid



Citric acid

Bernhaur in a series of three papers (48) gives a very excellent review of previous work on acid production with *Aspergillus niger* and a most interesting account of his own studies. He lists as the four essentials governing the direction and intensity of acid production:

1. Temperature.
2. Amount of usable foodstuff.
3. Chemical composition of food stuff.
4. Chemical reaction of medium.

Temperature changes vary the intensity of acid formation and the rate. With higher temperatures acids formed in the first phase of fermentation are further oxidized and at 37°C., the optimum temperature of growth, no acid accumulates. At 7°C., the minimum growth temperature, the greatest acid formation occurs but at a very slow rate. If oxygen is excluded or limited no acids are formed. There is no necessary parallel between the food needs for mycelial growth and the needs for acid production. He made his tests on fresh cultures on sugars containing nutrient salts and also grew mycelial felts on nutrient solutions and then tested their action on fresh sugar solutions. This plan has been followed by a number of workers and it is possible in commercial production to ferment three or four batches of sugar solution with the same mycelium and obtain excellent acid yields.

For mycelium growth he used a 10 per cent cane sugar solution containing 5 per cent peptone and 0.1 per cent each of acid potassium phosphate and magnesium sulphate, cultivating for five days at 30°C. For acid production he substituted a 10 per cent sugar solution with no additions and cultured for ten days at 25°C. He found that old

cultures formed more oxalic and less gluconic and citric acids. A free air supply lessens acid formation and an increase of surface area increases the same. The results in individual fermentations are a summation of factors involved. The formation of gluconic acid and citric acid are separate processes which may parallel, or one override the other.

Oxalic acid is formed later, after the sugar is used up probably by oxidation of gluconic acid.

Gluconic acid is formed by oxidation of the sugar but citric acid formation is in some way related to the nitrogen metabolism. Maze (11) thought that the fungus formed carbon dioxide and alcohol which later was used in formation of mycelial protoplasm. This on the autolysis of dead hyphae formed citric acid and nitrogen for new hypha but, as previously indicated, later writers did not accept his theory.

The thickness of the fungus mat is dependent on the nitrogen richness of the medium and thin mats form more acid than thick ones. He used one strain producing very little gluconic acid unless calcium carbonate was present and another that produced only gluconic acid. Gluconic acid formation however will be discussed in detail in another section. After discussing the various theories advanced as to acid formation and after detailing the results of his own experiments he gives the following conclusions:

Thin mats with low nitrogen supply and low temperature favor gluconic acid production.

Heavy mats with high nitrogen supply and higher temperature favor citric acid production.

Peptone or potassium nitrate nitrogen favors gluconic acid production.

Inorganic ammonium nitrogen favors citric acid production.

With very heavy mats no acid accumulates.

Hartman and Hillig (55) give a reliable method for the determination of citric acid as pentabromo-acetone. Other methods of analysing cultures for acids produced are given by Bernhauer (48), Currie (29), Butkewitsch (38), and others. Farbenid (56) has been granted a patent in Great Britain for the fermentation of materials in shallow layers flowing over inclined superposed trays with counter-current passage of air or oxygen and they consider this adaptable for citric acid production.

SUGGESTED PROCEDURE

From a consideration of the foregoing presentation of the various factors concerned in the production of citric acid from carbohydrates by mold fermentation the following methods of procedure are suggested for those undertaking to do this type of work.

If a strain of fungus known to be a good citric acid former is not available a number of brown or black molds of the Aspergillus and Penicillium type should be assembled and the reaction on sucrose or dextrose tested. For this work use at least 50 cc. of 10 to 15 per cent sugar solution containing the nutrient salts as used by Currie (29). Inoculate with mold spores and hold at 28°C. for ten days and then test for amount and type of acid formed. For production or detail studies select a strain or strains that will form a maximum of citric acid with a minimum of gluconic and oxalic acid. Stock cultures or selected strains may be maintained on slants of a similar medium with 1.5 to 2 per cent agar. It need not be neutralized.

Acid tolerance of selected strains may be increased by growing successive generations in flasks for three or four days each on the above solution to which increasing amounts of either citric or hydrochloric acid are added until the mold grows well in the medium with a pH value between 2 and 3.

For production or large scale experimentation work flat pans or tray cultures are preferable to flasks as they afford greater surface area for mycelial formation and a freer air supply.

For first fermentations of pure sugars it is essential to add the nutrient salts as indicated. Unless fermentations are started at a pH value of 3 or under as suggested by Currie (29), and in the Fernbach and Yuill (58) patent, solution should be sterilized at 15 pounds pressure for fifteen minutes or more. If the high acid values are used a half hour exposure at 100°C. will suffice and also it will not be so essential to protect the pans from accidental air contaminations.

After about ten days, or when titratable acidity has ceased to increase, the substrate may be syphoned off and replaced with pure sugar solutions. This may be repeated, using the same mat mycelium to ferment three or four batches of sugar solution, or it may be possible to devise a continuous flow process with a series of superposed pans (56).

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CHAPTER 5

Gluconic Acid Production

HISTORICAL

One of the newest fermentations suggested as suitable for industrial development is the production of calcium gluconate and gluconic acid from dextrose.

The first report of the detection of gluconic acid as a product of fermentation was that of Molliard in 1922 (1) in connection with the production of citric acid by the mold *Aspergillus niger*. Later Bernhauer (2) developed a strain which in the presence of calcium carbonate yields gluconic acid almost exclusively. It was found that the Aspergillus fermentation of dextrose could be so modified by varying control conditions that oxalic, citric or gluconic acids would be the predominating end products.

ORGANISMS USED

Chemical methods of manufacture of this acid being unsatisfactory, the attempt to produce it by fermentation was felt well worth while. May, Herrick, Thom and Church having found that a number of Aspergilli and Penicillia would produce more or less gluconic acid made a comparative study of 172 strains of fungi including Aspergilli, Penicillia, Monilla and Mucors (3). No Monilla or Mucor was found to produce appreciable amounts, but a number of Aspergilli and Penicillia did. As a result of their studies they selected a *Penicillium luteum-purpurogenum*, variety *rubrisclerotium* (4).

CONDITIONS OF GROWTH

The organism has an optimum temperature for acid production around 25°C. Higher temperatures may give a rapid preliminary growth but in a few days the mycelium begins to sink into the sugary solution and on its complete submergence growth and fermentation cease (5).

For acid production a 25 per cent glucose solution has been found to give best yields. Experimental work by Herrick and May (4)

show that the following standard culture medium for fermentation gives best results:

	grams
Commercial glucose (91.5 per cent $C_6H_{12}O_6$)	200.00
Magnesium sulphate	0.25
Di sodium hydrogen phosphate	0.10
Potassium chloride	0.05
Sodium nitrate	1.00
Water to make	1000 cc.

A later paper (5) substitutes an equivalent amount of phosphoric acid for the sodium phosphate.

Different strains of the fungi varied in their ability to produce acid, and the best strain was selected from the results of preliminary fermentations in wide-bottomed wide-mouthed Erlenmyer flasks. Yields of acid ranging from 55 to 65 per cent of the theoretical have been obtained.

The acid is formed entirely within the cells of the mycelium, a quiescent mycelium being essential for fermentation. Each square meter of mycelium is capable of yielding 4 to 4.5 kgm. of acid in fourteen days. For maximum yields shallow pan fermentations are best with a ratio of 0.25 to 0.30 sq. cm. surface per cubic centimeter of medium. To bring more carbohydrate in contact with mycelium early experimental work in small shallow flasks fermentations arranged for subsurface stirring of the liquid by horizontal stirred blades. With low concentrations of glucose this was found to increase yields but with 20 to 25 per cent solutions no appreciable differences between stirred and unstrirred batches were noted, so stirring was discarded as introducing an unnecessary complication.

Good yields are obtained with pH values ranging from 3 to 6.4. The standard medium given above will be about 4.8.

SEMI-PLANT PRODUCTION

An interesting account of semi-plant scale production of gluconic acid is given by May, Herrick, Moyer, and Hellbach (5). Best results were obtained with high grade aluminum pans, using a rack containing seven pans 43 inches square by 2 inches deep. Glass pans or iron pans coated with a high grade resistant enamel can be used but are more expensive and not as durable. Lacquered iron pans were satisfactory at first but later seemed to develop toxic prop-

erties as they were acted on by the acid formed. The entire rack was insulated and protected from contaminations by a cheese cloth screen with observation holes and holes for tubes for filling the pans and for inoculation. There was a steam jet under the lowest pan and also a duct to supply filtered air for circulation around the pans. Cheese cloth protection was sufficient as bacteria and yeasts do not thrive under the conditions of this fermentation.

Stock cultures were maintained on wort agar slants and stored at 4°C. As suggested by Thom (6) Reddish's malt extract agar may be used as a satisfactory substitute for wort agar if wort is not available.

Dry malt extract, 100 grams, dissolved in 900 cc. of distilled water and adjusted to 8° Kaiser (saccharometer) by further addition of water, approximately 100 cc. Adjust reaction to pH value 1.5. Autoclave fifteen minutes at 15 pounds pressure, add 1.5 per cent agar, heat twenty minutes at 15 pounds, filter through cotton, tube and sterilize fifteen minutes at 10 pounds or ten minutes at 15 pounds pressure.

To start fermentation cultures were transferred to slants of the following medium:

Bacto peptone, Difco	15.00
Commercial dextrose	30.00
Agar agar	30.00
Distilled water q.s.	1000 cc.

Ten days incubation at 25° to 30°C. gives good growth with heavy sporulation over the entire surface of the slant. Good ten-day growths are a light gray blue with white edges showing a trace of yellow. Spore dilutions from such tubes should be plated on agar of the same type periodically to study mutations and contaminations.

Three days before starting a batch 200 to 300 cc. flasks of the medium to be fermented were sterilized for fifteen minutes at 15 pounds pressure and then inoculated from the ten-day cultures. These were used to inoculate the large batches of 45 liters per pan. The solution for fermentation was sterilized at 100°C., cooled to 30°C. and poured into the pans which had been sterilized and cooled to 25°C. The pans were held at between 25° and 30°C. A film forms in two days and by the fourth day there was a rise of temperature up to 5°C. The fan was then started to supply filtered air to the rack at 23°C. and at a rate of 3 cubic feet per minute.

Acid formation was rapid. On the sixth day droplets of gluconic acid exudate began to form on the mold mycelium and by the tenth day these had increased to from 3 to 5 mm. in diameter. Pans were emptied on the eleventh day. The mycelial mat was pressed and extracted with hot water and the liquor added to the original liquid.

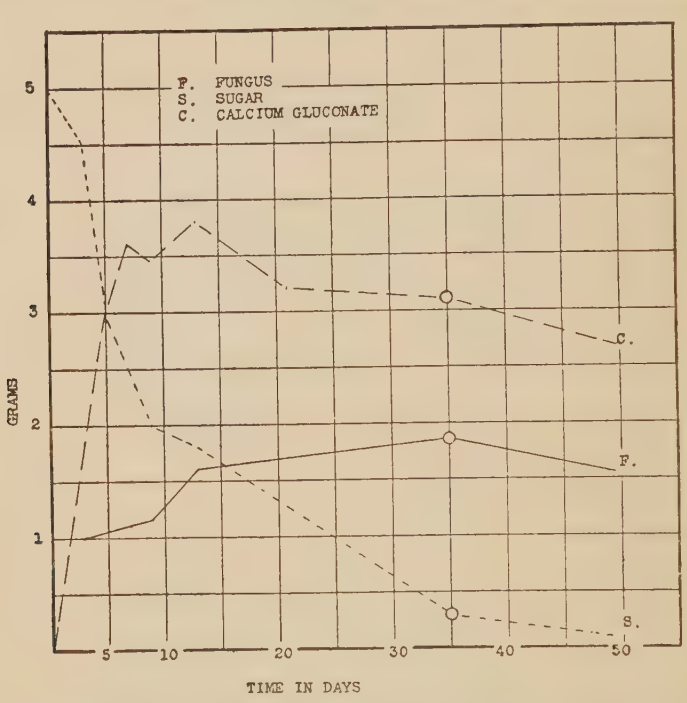

FIG. 4. CURVES SHOWING PRODUCTION OF CALCIUM GLUCONATE WITH UTILIZATION OF SUGAR AND WEIGHT OF THE FUNGUS
(After Bernhauer)

RECOVERY AND IDENTIFICATION

The resulting liquid was neutralized with calcium carbonate keeping at a pH value below 4.5 and evaporated to 50 per cent of its volume. The concentrate contained 25 to 30 per cent calcium gluconate which on cooling gelled to a mushy mass. This was separated by filtration or centrifuging and the filtrate cleared and reworked by addition to material for fresh medium.

The crude gluconate is dissolved in water to a 25 per cent solution, heated to boiling, filtered, cooled to crystallize and centrifuged. The resulting crystals are washed in ice water and dried at 80°C.

Cost of production was figured at about $10.00 per pound. Gluconic acid can be prepared from the crystals by quantitative treatment with sulphuric acid.

Herrich and May (5) have recently patented a process for the manufacture of gluconic acid from sugars and starchy substances by fermentation with *P. citrinum*, *P. divaricatum* and *P. luteum purpurogenum*, as well as related species.

The accompanying graph from Bernhauer (2) shows the ratio of acid production to fungus growth and sugar consumption as determined in his experimental work using a strain of *Aspergillus niger* (Fig. 4).

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CHAPTER 6

Lactic Acid Production

HISTORICAL

Lactic acid was found in cow's milk by Scheele (1). After three weeks fermentation the protein was separated and calcium hydroxide was added to precipitate the phosphates; the calcium lactate remained dissolved and was decomposed with oxalic acid. The filtrate was concentrated and taken up with alcohol. After concentrating the alcohol, the lactic acid was obtained as a thick syrup which could not be distilled or crystallized. From the first work as well as other work Scheele concluded that the acid was free from the acetates and that a new acid resulted, while prior to 1808 most chemists had considered it as a degenerate acetic acid.

Lactic acid is probably the oldest known acid. Gay-Lussac (2) investigated lactic acid in 1833. In 1850 Rowlaudson (3) claimed that the souring of milk was an oxidation process. Pasteur was the first to describe an organism which would ferment sterile milk. Lister, by the tedious dilution method, isolated a lactic acid bacillus.

Technical lactic acid was first produced by an organism known as *Bacillus acidificans longissimus* (7), probably identical with *Lactobacillus delbrucki* growing at an optimum temperature of 50°C. Keeping silage at 50°C. promotes the growth of this rod and inhibits the butyric acid formers. It is sometimes used in breweries to make sour mash.

A slow decomposition of sugars may be chemically accomplished in alkaline solution in the sunlight. With sodium or potassium hydroxide the end products are ethanol and carbon dioxide. With calcium hydroxide the end product is calcium lactate.

GENERAL CONSIDERATIONS

Fermentation lactic acid is alpha-hydroxy-proprionic acid $CH_3CHOH \cdot COOH$ (27). It has an asymmetric carbon atom and therefore exists in two optical isomers. Industrial lactic acid is prepared by the fermentation of various sugars, chiefly dextrose, maltose and lactose. Many organisms accomplish the fermentation, produc-

ing various yields and giving various by-products. Some produce gas and volatile acids.

The industrial starting point may be potato starch, corn starch, stale bread, molasses or whey or any fermentable carbohydrate. The starch is hydrolyzed with dilute sulphuric acid and the same acid is used to free the lactic acid from the calcium lactate produced.

Most economical results are given by starting with a 12 per cent sugar solution, which neither inhibits growth nor wastes sugar. Whey contains from 4 to 5 per cent lactose and must be a by-product industry in cheese manufacturing in order to pay as a source of acid.

The lactic acid must be neutralized as formed or it will shortly inhibit growth. It may be continually neutralized by the preliminary addition of an excess of calcium or zinc carbonate. The pH value hereby remains constant but, due to the presence of CO_2 in solution, the process is consequently slow, requiring about twenty days.

Many by-products are formed in the lactic acid fermentation, as acetic, formic and butyric acids, alcohols and carbon dioxide. The proportions depend upon the organism, the medium and the temperature.

With *Streptococcus lactis* quickest results are obtained between 30° and 35°C., but better yields are obtained at a lower temperature. This is illustrated by recent work upon three strains:

TEMPERATURE	A	B	C
°C.	*per cent*	*per cent*	*per cent*
37	0.89	0.87	0.6
30	1.00	0.96	0.81
18	1.08	1.06	0.88
6	0.7	0.73	0.62

This is probably due to the free accumulated acid being more inhibitive at the higher temperatures. Fluctuation in temperature probably improves the yield of lactic acid. Lactogens naturally inhibit the growth of other non-acidophilic organisms.

POLARITY OF FERMENTATION LACTIC ACIDS

The literature concerning the optical activity of fermentation lactic acids consists largely of contradictions (7). It is a general rule that

optically active substances cannot be synthesized by purely chemical means from inactive substances but nature prefers to create active isomers. Light is thrown upon the method by which this result is accomplished in the lactic acid fermentation by preparing a medium consisting of peptone and inactive calcium lactate together with the necessary salt. Add to this flask an excess of calcium carbonate and sterilize. Inoculate with a small loopful of a lactic acid organism which has been cultivated in a medium containing calcium lactate. A fermentation will ensue in which one or other of the optical isomers is broken down. After fermentation for instance, with *B. coli*, the medium will be found to contain *l*-calcium lactate. This may be ascertained by filtering off the excess of calcium carbonate and evaporating the filtrate to a small bulk which may be read in a polarimeter.

The fact that so many organisms are reported to yield only racemic acid is accounted for by the ease with which an active lactic acid is converted into a racemic mixture. It has been assumed by some investigators that no single organism can produce a pure racemic acid, but *d-l* mixtures may be formed.

The following list has been collected from various sources and is advanced tentatively:

Strept. lactis	*d*-acid predominates
Strept. pyogenes	*d*-acid predominates
B. lactis aerogenes	*l*-acid predominates
Lactobacillus caucasicus	*l*-acid predominates
L. boas-opperli	*l*-acid predominates
L. delbrucki	*l*-acid predominates
L. lactisacidi	*l*-acid predominates
L. cucumeris	*l*-acid predominates
L. bulgaricus (4 strains)	Definite contradictions
V. cholerae	*l*-acid predominates
B. typhosum	*l*-acid predominates
Sp. denecke	*d*-acid predominates
B. coli	*d*-acid predominates
Pencillium glaucum	*d*-acid predominates

If the theory of the utilization in metabolism of one optical modification is true, it should follow that the closer our yields approach 100 per cent the more nearly racemic will be the acid and that any yield over 50 per cent must, if optically active, be a predominance of one form and not a pure dextro nor a pure laevo acid.

ACID YIELDS

Lactic acid yields do not run over 90 per cent. There are three possibilities to consider in explaining the loss in yield.

1. The break down to lactic acid is incomplete.
2. The lactic acid produced has gone into other combinations.
3. Of the lactic acid produced part (perhaps, one modification) has been further decomposed.

REQUIREMENTS FOR HIGH YIELDS

Fermentation by lactic acid bacteria at the optimal pH value 6.2, is accelerated 50 to 60 per cent by the addition of phosphate, the optimum concentration of the latter being 1 per cent P_2O_5 for both *B. casei* and *Strept. lactis* (23).

Lactic acid bacteria can grow only in the presence of proteins or amino-acid complexes. They do not reduce hydrogen peroxide or nitrates, and are definitely Gram-positive. In a classification of lactic acid bacteria the chief groups form only traces of accompanying substances while the lesser groups form with the lactic acid considerable quantities of gas and other substances (18).

To obtain the maximum amount of lactic acid compounds in the medium it is necessary to add a neutralizing agent on the second day of inoculation (15). Fermentation proceeds until very little sugar remains in the medium. The lactates formed do not materially hinder the process and by a continuous neutralization the vessel may be filled with the solid lactates of magnesium or zinc.

Co-zymase is an essential factor in lactic acid fermentation. The dried preparation of bacteria is inactivated by washing with water but reactivated by the addition of the boiled extract. Esterification of the sugar with phosphoric acid occurs with *Streptococcus lactis*. Since zymophosphate formation has also been demonstrated in propionic acid fermentation, it is probable that with various other fermentations the cleavage of sugar is inaugurated by a sugar-phosphate esterification (19).

PROPERTIES OF LACTIC ACID

Lactic acid is a colorless, odorless, somewhat viscous liquid having a strongly acid taste and containing about 10 per cent of water. By distilling the aqueous solution under a very low pressure lactic acid can be purified and obtained as a crystalline solid melting at 18°C.

When it is heated at ordinary temperature in an attempt to remove the water it is partially converted into the anhydride before dehydration is complete.

The acid may be obtained from disaccharides or monosaccharides and is one of the most common products of bacterial activity, but often merely as a by-product, as in the alcoholic fermentation.

LACTIC ACID FORMING ORGANISMS

Those organisms forming lactic acid may be divided into two main groups, namely, those that produce only lactic acid or predominatingly so with only traces of other by-products, (8) the fermentation being represented by the following equations:

$$C_{12}H_{22}O_{11} + H_2O = 4\ C_3H_6O_3 \text{ from a disaccharide}$$

and

$$C_6H_{12}O_6 = 2\ C_3H_6O_3 \text{ from a monosaccharide}$$

Secondly those producing other and various by-products as do Escherichia coli and others of the colon group.

$$2\ C_6H_{12}O_6 + H_2O = 2\ C_3H_6O_3 + C_2H_4O_2 + C_2H_5OH + 2\ CO_2 + 2\ H_2$$

The organisms of this and other groups producing many and varied by-products in appreciable quantities do not concern us in this chapter.

LACTOBACILLUS FERMENTATIONS

Among organisms producing only or largely lactic acid two types are of interest for our purposes, the Lactobacilli, to which belong *L. leichmanni*, *L. delbrucki*, *L. acidophilus*, *L. casei* and *L. bulgaricus* and the pentose fermenters discussed under xylose fermentation and the *Streptococcus lactis* or *Streptococcus acidi lactici*.

L. delbrucki (29) as previously stated is probably identical with *Bacillus acideficans longissimus*, the first organism used to produce technical lactic acid (7). It is used frequently in European breweries to prepare sour mash to encourage yeast fermentation and discourage butyric acid fermenting contaminants.

L. leishmanni (19) and *L. bulgaricus* are used singly for lactic acid production and *L. delbrucki* in conjunction with *L. bulgaricus* or *Streptococcus lactis* (9). *L. bulgaricus* alone is suggested in one Amer-

ican patent (11). Where whey is the raw material to be fermented *S. lactis* (29) is probably the organism of choice, though the others may be used.

Where the Lactobacilli are used fermentations are conducted at or near 50°C. or with *L. bulgaricus* possibly as low as 40°C. These organisms are microaerophilic and this with the high optimum temperature aids in the maintenance of pure culture fermentations.

LIMITING FACTORS OF FERMENTATION

Under the best of conditions with any of the lactic acid formers used for lactic acid production actual yields may be from 90 to 98 per cent of the theoretical, from 2 to 10 per cent of carbohydrate being used for cell growth and by-product production which never can be entirely eliminated. For routine work probably any yields of over 70 per cent of sugar converted to lactic acid may be profitable if the raw material is cheap. The enzyme producing lactic acid has not yet been isolated.

Rogers and Whittier (26) have discussed at some length certain factors limiting lactic acid fermentations. In the normal period of lactic fermentations the bacterial growth passes through four stages, the lag period, the period of rapid or logmarithmic growth, the period in which there is little or no increase in numbers, and the final period in which fermentation ceases and the cells slowly die. The changes are comparatively rapid in going from the first to the second and from the second to the third periods. They are accompanied by morphological and physiological changes in the cells. The transition from the third to the fourth period is a gradual one.

The fermentation activity follows the population activity closest through the period of rapid growth, but if the effect of the acid is removed by neutralization the fermentation activities continue after the increase in population has ceased, though the same factors appear to limit both growth and acid production.

Among the causes most frequently suggested for limitations of the bacterial growth are the exhaustion of the food supply and the accumulation of the end products or by-products of growth. Ordinarily, however, growth is arrested before the available food supply is exhausted. Control of the pH value permits the attainment of a greater bacterial population than when the accumulating acid is not neutralized.

Cornwell and Beer (21) have shown that in filtered cultures reinoculated with the same organism the population does not reach the numbers attained in the first propagation. This is an indication that the inhibiting factor is carried in the filtrate and not directly concerned with the cells. Obviously acids are not the only inhibiting factors formed, and the minor products of metabolism, as aldehydes, may have a definite inhibiting effect.

Aeration of a culture maintained at a constant pH value had the effect of a further prolongation of the fermentation and an increase in the bacterial population. One patent (22) takes advantage of this fact and claims that aeration also lessens likelihood of butyric fermentation from contaminants developing. Of course this could not be used with the microaerophilic Lactobacilli. That stimulation was not due to removal of volatile substances with the air bubbles was shown by Rogers and Whittier by freezing out the volatile matters in a condenser and adding them to a fresh culture with no inhibitory effect. They conclude that concentration of undissociated lactic acid is the principal factor in limiting fermentation.

In other experiments they showed that decrease of lactate concentration after the attainment of a condition limiting multiplication and growth, enables the medium to support again the activities of *Streptococcus lactis*. The lactates were removed by fermentation with *B. casei*.

Van Dam (12) showed that bacterial activity ceases at lower hydrogen-ion concentration when the acid added is lactic acid, either free or as a salt. They also found that the value which is constant under limiting conditions in the presence of added lactates is not hydrogen-ion concentration but the concentration of the undissociated acid.

MIXED FERMENTATION

Among the Lactobacilli three have been used for producing technical lactic acid. *Lactobacillus delbrucki* will not ferment lactose so could not be used with whey but it will ferment dextrose, levulose, galactose, maltose, sucrose, and dextrin. It produces laevo-rotary lactic acid, forming up to 1.6 per cent acid in unneutralized mash. Friedberger's United States patent (9) calls for the fermentation of dextrose, grape sugar, glucose, starch sugar, anhydrous sugar and the like. It is stated that *L. delbrucki* normally, quickly and completely,

converts maltose into lactic acid but only slightly acidifies dextrose. The bacilli are first grown for two days in maltose at 49° to 51.6°C., and then gradually accustomed to dextrose by daily additions for three days of about one-fourth volume of 10 per cent dextrose. It will then ferment dextrose actively. For commercial production a 10 per cent dextrose solution is boiled with steam for one hour, cooled to 48°C. and inoculated with the trained culture. Fermentation continues for two days with intermittent or constant agitation in the presence of sufficient sterilized chalk liquor to bind all the lactic acid theoretically expected (90 per cent of the value of sugar used). To complete conversion of sugar to acid the mash is seeded with cultures of *L. acidi-lactici* and *L. bulgaricus*.

MALTOSE FERMENTATION

Orla-Jensen (18) states that maltose is frequently used for lactic acid production. A 10 to 20 per cent maltose prepared by saccharifying starch with malt extract, or Koji malt, or maltase from Aspergillus could be used, see chapter on maltase production. Chalk is added and the solution sterilized, cooled and inoculated with a lactobacillus, *L. delbrucki* or *L. leichmanni* may be used, and fermented for one week at 50°C. As calcium lactate forms, the solution gradually sets to a pasty mass of crystals which is pressed and decomposed quantitatively with sulphuric acid. The calcium sulphate is filtered off and the acid concentrated by evaporation.

LACTOBACILLUS LEICHMANNI FERMENTATION

The granulated high acid forming bacteria of cereal infusions are closely related to *Lactobacillus delbrucki*. Their cultural characteristics and fermentations place them in the species described by Henneberg under the name *Lactobacillus leichmanni*. Glucose, fructose and mannose are readily fermented. The fermentation of galactose is somewhat slower. Disaccharides are attacked to a lesser degree and trisaccharides scarcely at all. Considerable acid is formed from glucosides and from dextrin. Levo-rotatory lactic acid is the major product of the fermentation. Traces of volatile acids, alcohol and carbon dioxide are found (20). This organism may be used in a similar way to *L. delbrucki* and it may also be used to ferment lactose. It is microaerophilic with an optimum temperature of 45°C.

MALTED STARCH FERMENTATION

The first United States patent granted (5) in 1907 for the lactic fermentation was for manufacturing lactates, presumably by a lactobacillus fermentation though the organism was not named. Fermentation started with a 10 per cent sugar solution from malted starch. Calcium carbonate was used for neutralization and the fermentation was conducted between 49° and 57°C. Fresh 50 per cent glucose was added from time to time as fermentation progressed so as each time to bring sugar concentration back to 10 per cent. By this means 20 per cent of sugar could be converted into nearly 20 per cent lactic acid which would not need concentration with its resultant darkening. Recovery was as previously described.

PURIFIED WHEY FERMENTATION

In the same year a patent was granted to Just (6) in which he started with whey or crude lactose as a raw material. To this was added a mineral absorbent, as infusorial earth, talc or asbestos which on evaporating the whey entrains the fat and undesirable solids. When the dried mass is leached the milk sugar and some soluble amides and peptones and milk salts dissolve out to form a fermentable solution. Drying is done over a revolving drum. For fermentation a rennet solution is added which is stated to be rich in active lactose fermenters as fermentation is to be conducted at a temperature between 27.7° and 41°C., probably *Streptococcus lactis* is depended upon.

VEGETABLE IVORY FERMENTATION—L. BULGARICUS

Saxe (11) has been granted a patent for manufacturing lactic acid by fermentation of a mannose solution obtained by hydrolysis of ground vegetable ivory nuts with 3 per cent of sulphuric acid to double the volume of nut meal at boiling temperature for six hours. This gives a 15 to 30 per cent mannose solution which is diluted to 10 to 15 per cent after neutralizing with calcium carbonate and filtering. *Lactobacillus bulgaricus* is used to ferment the liquor after it has been treated with an excess of calcium carbonate or zinc oxide sufficient to neutralize the acid formed.

HYDROLYZED SAWDUST FERMENTATION

In 1919, Richter (14) was granted a patent to produce lactic acid by fermentation of sawdust hydrolyzed with 8 parts of a 0.5 per cent

lactic acid to 1 part sawdust at 150 pounds pressure for two hours. The liquor is filtered, calcium carbonate added and it is then inoculated with lactic acid bacteria.

FERMENTATION WITH AERATION

Faithful (22) was granted a patent in 1926 for lactic acid manufacture from hydrolyzed carbohydrate containing small amounts of protein and potassium and other nutrient salts. The hydrolyzed liquor is diluted to 12 to 15 per cent sugar and adjusted to 0.3 to 0.8 per cent lactic acid acidity. The liquor is inoculated with a pure culture of lactic acid formers and fermented at 33° to 35°C. During the entire fermentation period air is bubbled through at the rate of 15 cubic feet of air per 1000 gallons of solution. Every eight hours sufficient milk of lime is added to reduce the increasing acidity to 0.65 per cent lactic acid. Rather complicated directions then follow for recovery of the lactic acid which is obtained as a light colored solution or of lactates. The purpose of the aeration has been discussed previously.

CASEIN FROM WHEY

A large amount of the casein now used in the manufacture of adhesives, paints and plastics is produced by lactic acid fermentation of skim milk by the genus Lactobacilli. Milk from which cream has been separated is allowed to flow in large vats and held at about 50°C. until the curdling is complete due to the increase in acidity. The relative composition of cow's milk, which is the largest source of casein is shown.

	per cent
Water	87.3
Casein	3.0
Albumin	0.5
Fat	3.5
Lactose	5.0
Ash	0.7

The curd which separates out is broken by agitation and the clear fluid, or whey, is run off. It is then washed with several portions of clean water and after draining the excess water expelled with moderate pressure. The resultant curd is usually a buff color. The waste whey can be used for a chicken food.

LACTATES FROM WHEY

In September, 1929, Robinson was granted a patent (28) for production of lactic salts from whey, by fermentation with either *Streptococcus lactis* at between 23° and 24°C. or *Lactobacillus bulgaricus* at 40.5°C. with periodic neutralization with sodium hydroxide. The resulting sodium lactate solution is concentrated in vacuum to remove coagulable proteins, filtered and used as an emuslifying or fluxing agent in the preparation of pasteurized cheese.

Certain bacteria, which occur in sour milk and beet juices, possess the property of inverting saccharose before subjecting it to lactic fermentation. The name suggested for this group of organisms is that of *Bacillus saccaro invertenti* and the group is subdivided into inverting-lactic bacilli, *Bacillus invertenti lactici*, and inverting-acetic bacilli, *Bacillus invertenti acetici* (25).

With *B. invertenti lactici:* 100 grams of saccharose yield 60 to 80 per cent lactic acid, 10 to 20 per cent acetic acid, 1 to 7 per cent alcohol and traces of higher acids.

With *B. invertenti acetici*, which is even more active: 100 grams of saccharose yield 40 to 50 per cent acetic acid, 1 to 20 per cent lactic acid, 10 to 20 per cent ethyl alcohol and 1 to 2 per cent acetone.

In the commercial manufacture of lactic acid by lacto-inverting bacteria, it was observed that the deposit which settled out on allowing 50 per cent lactic acid to stand for some months consisted of practically pure mannitol. Investigation of several strains of bacteria showed yields, on 100 grams of sucrose, of 1.1 to 79.1 per cent lactic acid and 0.0 to 17.85 per cent mannitol, maximum mannitol yield being obtained with 42.5 per cent lactic acid yield. Experiments in the symbiosis of lacticomannitic ferments with the mannitic ferments of wines did not give an improvement in yields (25).

STREPTOCOCCUS LACTIS FERMENTATION

Orla-Jensen (18) and Hammer (27) both state that *S. lactis* produces *d*-lactic acid. A study of many cultures of *Streptococcus lactis* shows that important differences occur among them, thus indicating that they should be looked upon as a group of closely related varieties or species. The differences may include flavor, aroma, and ropiness of end products, reducing power, coagulation and temperature requirements. The *S. lactis* is defined by Hammer (27) as an organism coagulating milk rapidly or fairly rapidly with reduction of litmus

but without digestion of casein or formation of gas, and which appears in milk as a Gram-positive coccus arranged in chains and pairs. Coagulation occurs at a more or less definite acidity depending upon the composition of the milk and various other factors.

Most of the group produce acid in sufficient quantity so that coagulation is very definite and quick. However, some organisms placed in this group coagulate milk more slowly.

Litmus reduction is influenced by coagulation as the typical white curd with a pink top is only seen where coagulation occurs.

The *Streptococcus lactis* organism (29) first discovered by Lister (4) is especially suitable for fermenting whey to produce lactic acid. It may appear as oval or slightly rod shaped in pairs or chains. It usually is about 0.5 by 1 micron in size. Its optimum growth temperature is 30°C. and the thermal death point of different strains may range from 60° to 70°C. It will ferment lactose readily at from 20° to 30°C. though its optimum growth temperature is over 30°C. It grows rapidly under favorable conditions but produces minute colonies on agar. It coagulates milk in twenty-four hours with the production of 3 per cent or more of lactic acid. It is easily isolated on milk agar from sour milk in which it will make up from 90 to 100 per cent of the total flora at room temperature.

The senior author has found that sweet whey from clean milk may be satisfactorily fermented without further addition of bacteria, thus avoiding the necessity of maintaining pure cultures. We have fermented whey in 5-gallon lots after concentration in vacuum to a lactose percentage of approximately 12 per cent. Fermentation was conducted at from 20° to 22°C. with twice daily neutralization with milk of lime. With periodic neutralization with calcium hydroxide fermentation was complete in seven or eight days with little residual lactose, whereas with the addition of calcium carbonate at the start and periodic stirring fermentation was much slower, up to eighteen days, and acid yields were lower. By this method we have prepared a good grade of light brown acid with a yield equivalent to over 70 per cent of the lactose present.

Instead of concentrating the whey before fermentation it would be possible to raise its sugar content with crude lactose as in the Just patent (6).

PERIODIC NEUTRALIZATION

Although it is frequently stated that the use of calcium carbonate as a buffer will maintain the pH value at approximately 7, or the neu-

tral point, it has not done so in our experience, we believe owing to the liberated carbon dioxide in solution.

We tentatively explain the apparently stimulating action of periodic neutralization with milk of lime as due to the fluctuation of the pH value repeatedly causing the development of new generations of more vigorously fermenting bacilli. We are inclined to believe that the conception of our ideal optimum pH value or temperature condition for bacterial activity may be as faulty as the idea of a fixed optimum climatic condition for the human organism. According to Ellsworth Huntington (10) man prefers a fluctuating environment with considerable variation between two fairly well defined limits. Bacteria may also prefer such fluctuations over an optimum temperature and pH range rather than any one fixed environmental condition. It is well recognized that higher plants prefer fluctuation to stagnation in environment.

Where periodic neutralization is used, however, care must be exercised to avoid shifting the pH value to the alkaline side, especially with previously unsterilized substrates. In our early efforts in this line such over-adjustment permitted the development of a butyric fermentation much to our disgust.

We are aware that Peterson and Fred and Davenport did not find any more rapid or complete xylose conversion with periodic neutralization than with preliminary carbonate introduction (13). The progress of fermentation may be checked by sugar determinations (17) to find the amount of unconsumed substrate, and by colormetric or electrical pH determinations to show progressive acid production. The lactic acid yields are determined by the usual method for the determination of non-volatile acids. An aliquot portion of the fermented substrate is acidified with strong hydrochloric acid, evaporated to dryness on a water bath, taken up in water and re-evaporated several times to drive off all volatile acid, diluted to the original volume and titrated with N/10 sodium hydroxide. Copper vats are not suitable for milk or other acid fermentations owing to the antiseptic action of the copper, as shown by Springer (16).

COMMERCIAL LACTIC ACID

Lactic acid is marketed as 20 per cent light or dark acid, as 40 to 50 per cent acid and as 88 to 90 per cent chemically pure. If edible acid is to be prepared the sulphuric acid used in conversion must be

arsenic-free. As many of the lactobacilli might be used to produce lactic acid we give a synopsis of the tribe taken from Bergey (29) and including optimum temperatures, carbohydrates acted upon, per cent of acid formed and type where listed and source from which isolated.

Lactobacillaceae: Rods, often long and slender, Gram-positive, non-motile, without endospores. Generally produce acid, as a rule lactic, from carbohydrates. When gas is formed it is CO_2 not H_2. Usually somewhat thermophylic. Surface growth on media, poor. Microaerophilic.

A. Produce only traces of by-products other than lactic acid.
  a. Acid in lactose.
  b. Action on sucrose unknown.
  c. Action on mannitol unknown.
    1. *L. caucasius.* Acid in dextrose and lactose O.T. 37° to 40°C., mostly *l*-acid, 1.0 to 1.6 per cent in milk, from kefir.
    2. *L. boas-oppleri.* Acid in dextrose and lactose O.T. 40°C., *l*-acid, 1 per cent in milk, gastric contents.
  cc. Acid in Mannitol
    3. *L. bulgaricus.* Acid in dextrose, levulose and galactose. O.T. 40° to 45°C., 2.7 to 3.7 per cent *l*- or inactive acid in milk, from yoghurt.
  bb. Acid in sucrose.
  c. No action in mannitol.
  d. No action on maltose.
    4. *L. helveticum.* Acid in dextrose, levulose, galactose and a little in dextrin. O.T. 40° to 43°C., from sour milk and cheese.
    5. *L. thermophilus.* Acid in dextrose, amylum and glycerol. O.T. 50° to 62°C., from pin point colonies in pasteurized milk.
  cc. Acid in mannitol.
  d. Acid in maltose.
  e. No action on raffinose.
    6. *L. leichmanni.* Acid in levulose, dextrose, mannose, trehalose, salicin, glycerol, slightly in galactose and mannitol. O.T. 45°C., 1.3 per cent acid in mash, from sour mash and sour milk.
    7. *L. pabuliacidi.* Acid in dextrose and glycerol. O.T. 34° to 40°C., 4 per cent acid in dextrose, from beet mash and cheese.
  ee. Acid in raffinose.
  f. No action on arabinose.
    8. *L. acidophilus.* Acid in dextrose, levulose and galactose. O.T. 37°C., from intestinal canal.
    9. *L. listeri.* Acid in dextrose, levulose, galactose and trehalose, little in dextrin, O.T. 34°C., 1.1 per cent acid in mash, from sour mash.

10. *L. lactisacidi.* Acid in dextrose, levulose, galactose, dextrin, less in raffinose, mannitol and quercit. O.T. 40°C., *l*-acid formed from milk.
11. *L. cucumeris.* Acid in dextrose, levulose, glactose, dextrin and trehalose, little in dextrin, O.T. 34°C. *l*-acid from pickles.

ff. Acid in arabinose.

12. *L. plantarum.* Acid in dextrose, levulose, galactose, mannose, dextrin, imulin, salicin and sorbitol. O.T. 37°C., from sour dough, cheese, pickled cabbage.
13. *L. wortmanni.* Acid in dextrose, levulose, galactose, dextrin, trehalose and *a*-methyl-glucoside. O.T. 34°C., from sour mash.
14. *L. busaeasiaticus.* Acid in dextrose, levulose, galactose, mannose, dextrin, inulin and amylum. O.T. 32° to 42°C., 1.1 per cent acid, from Busa.
15. *L. pentosus.* Acid in dextrose, galactose, mannose and *a*-methyl-glucoside. O.T. 30°C., from saurkraut.
16. *L. arabinosus.* Acid in dextrose, levulose, galactose, mannose and melizitose. O.T. 30°C., from saurkraut.

aa. No action on lactose.

b. Acid in sucrose.

c. No action on dextrin.

17. *L. beijerincki.* Acid in dextrose, levulose and galactose. O.T. 35° to 45°C., from sour potato mash.

cc. Acid in dextrose.

18. *L. lindneri.* Acid in dextrose and levulose. O.T. 27° to 32°C., 0.9 per cent acid, 1.5 per cent alcohol from spoiled beer and distillery yeast.
19. *L. berolinensis.* Acid in dextrose, levulose and galactose. O.T. 21° to 24°C., 1 per cent acid in mash, from spoiled beer.
20. *L. delbrucki.* Acid in dextrose, levulose and galactose. O.T. 30° to 35°C., *l*-acid, 1.6 per cent acid in mash from sour potato mash.

B. Produce considerable amounts of by-products other than lactic acid. 15 species listed.

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CHAPTER 7

Propionic Acid Production

As early as 1854 Strecker (1) demonstrated the presence of propionic acid in jars containing a mixture of sour milk, cheese, grape sugar, chalk and water which had been standing in his laboratory for over a year. In 1907 Freudenreich and Orla-Jensen (2) found that propionic acid production in Swiss cheese was due to a bacillus, named by them *Bacillus acidi propionici*, *a* and *b*, which they isolated from Swiss cheese, sour milk, and limberger cheese. These organisms fermented lactic acid or calcium lactate to form propionic and acetic acid, carbon dioxide and water. The free carbon dioxide formed being responsible for the holes in the cheese. They gave the following formula:

$$3\ C_3H_6O_3 = 2\ C_3H_6O_2 + C_2H_4O_2 + CO_2 + H_2O$$

In 1921, Sherman (5) confirmed this work, demonstrating that organisms of this type are responsible for "eyes" and flavor of Swiss cheese. He and his associates have since then conducted extensive studies on this group of bacteria and on their fermentation reactions, and have suggested their use for the commercial production of propionic and acetic acid and their ketones from whey as a by-product industry, and have taken out three patents thereon, all of which are assigned "to the people of the United States" so that no one may establish a monopoly of the process if it prove commercially feasible. Sherman and Shaw found that the presence of certain other organisms, as *Streptococcus lactis* and *Lactobacillus casei* would apparently increase propionic acid production. This may be due to the increased production of lactic acid which is utilized by the propionic organisms. Later (8) they found that some proteolytic and alkali producing organisms such as *Proteus vulgaris* and *Bacillus alkaligenes* also appeared to stimulate the growth of the propionic organisms and to increase acid yields.

In experimental work they employed sterilized whey fermented in the presence of calcium carbonate, inoculating with 1 per cent of pure cultures of an organism isolated by Sherman and designated as

B. acidi propionici, d. They fermented for ten days at about 30°C. At the end of this time the whey was filtered, concentrated in vacuo, the volatile acids liberated with sulphuric acid and steam distilled. They found acetic and propionic acids to be produced in a ratio of approximately one to two and together equivalent to 66.56 per cent of the sugar present. The optimum lactose concentration for fermentation was found to be from about that of whey up to 8 per cent.

The first patent of Sherman and Shaw (6) calls for the use of the associated organisms *Proteus vulgaris* and *Bacterium alkaligenes* to "purify" propionates produced by fermentation or otherwise. Their second patent (7), two months later, uses the same two associated organisms and also *Lactobacillus casei* to "accelerate" the propionic fermentation. While the third patent (10), five months later still calls for a pure culture fermentation with the *B. acidi propionici.* In the illustration given with this patent they use 1000 pounds of sterilized whey, 40 pounds of pulverized limestone and 40 pounds of a whey culture of of the organism. They ferment for ten days with frequent agitation. Then they filter, concentrate to 150 pounds, add 66° Be sulphuric acid until the mass reacts blue to Congo red paper and steam distill the mixed acids. Mixed ketones can be prepared from the mixed acids by converting to alkali metal salts, evaporating to dryness and distilling at 400°C. The ketonic mixture obtained may be fractionally distilled.

Whittier and Sherman (9) discuss the reaction and suggest a somewhat different procedure. Using a 1 cc. mixed culture of *B. acidi propionici* and *L. casei* grown for three days at 30°C. they inoculate 100 cc. sterile buffered whey (5 grams $CaCO_3$ bringing the whey approximately to the optimum value of pH 7). They incubate at 30°C. until fermentation is complete, or ten to twelve days. Then determine total acidity by titration with alkali, transfer to a 100 cc. graduated flask, dilute to the mark and centrifuge five minutes to clarify.

Take 50 to 70 cc. of the clear supernatant fluid in a 500 cc. round-bottom flask, made acid to Congo red with 10 per cent sulphuric acid, dilute to 250 cc. and stream distill 1000 cc., keeping the flask at a constant volume. An aliquot is titrated with 0.05 N NaOH to determine mixed volatile acid. Then a volume equal to 28 cc. of 0.05 N NaOH is made alkaline to phenolphthalein, evaporated to 60 cc. transferred to a 110 cc. flask, made acid to Congo red, diluted to the 110

cc. mark and subjected to a Duclaux (3) distillation to determine ratio of propionic to acetic acid. In larger fermentations they obtained 2.4 pounds of propionic and 1 pound acetic acid from 5 pounds of lactose. They determined that with inoculum 5 per cent by volume of whey and a twelve-day incubation they could convert 85 per cent of the lactose to the mixed acid.

The propionates and acetates could be converted to free acids or concentrated and distilled at high temperature to yield a mixture of 20 per cent acetone and 40 per cent each of methyl ethyl and diethyl ketones.

TABLE 3

THE FERMENTATION OF PENTOSES BY PROPIONIBACTERS

	VOLATILE ACIDS	NON-VOLATILE ACIDS	CARBON DIOXIDE
	per cent	*per cent*	*per cent*
A. Arabinose	30	25	15
Xylose	40	50	10
B. Arabinose	70	20	10

In the arabinose fermentation the ratio of propionic acid to acetic acid is 1 to 9.

Werkman and Kendal (14) state that the propionobacters cannot use ammonium salts as a source of nitrogen. Vegetable extract may stimulate growth.

Foote, Peterson and Fred (13) investigated the fermentation of pentoses by propionic organisms, twenty cultures being tested for their ability to ferment arabinose and xylose, of these thirteen fermented the former and but one the latter. One culture fermented both pentoses. The results with these cultures on the two sugars is given in table 3.

Van Neil (12) made an extensive study of the propionobacters and published a monograph on the same. In anaerobic fermentations the volatile acids are propionic and acetic and CO_2 with very small amounts of succinic acid. He recognizes a double fermentation reaction in the fermentation of lactic acid. One molecule of lactic acid is oxidized to acetic acid and CO_2 with the formation of hydrogen.

$$C_3H_6O_3 + H_2O = C_2H_4O_2 + CO_2 + 2H_2$$

The hydrogen formed in this reaction is used to reduce two molecules of lactic acid to propionic acid.

$$2C_3H_6O_3 + 2H_2 = 2C_3H_6O_2 + 2H_2O$$

Van Neil says that the small amounts of succinic acid found originate from the nitrogen compounds. He regards pyruvic acid as an intermediate.

As far as we are able to learn but one member of this group the *B. acidi propionici, d,* of Sherman has been used in semi-plant scale fermentation and we believe not even that has been employed successfully in full commercial production, though it may be so ere long.

Whittier and Sherman state that propionic acid produced as a residue from the refining of acetic acid is used by perfume manufacturers. Ethyl propionate has been patented as a pyroxylin solvent and propionyl cellulose may be a possible substitute for acetyl cellulose. The wide range of temperatures under which propionic acid remains fluid (from $-22°$ to 140°C.) may cause it to replace acetic acid as a solvent.

As there are possibilities that other members of this group may be useful industrially we give the synopsis of the group given in Bergey's Manual (15).

Propionobacterium: Non-motile, Gram positive rods, growing under anaerobic conditions in neutral media as short rods, and under aerobic conditions as long irregular rod shaped cells. Ferment lactic acid, carbohydrates and polyalcohols with the formation of propionic and acetic acids and CO_2. Complex organic nitrogen compounds are required for development. Catalase formed.

1. Non-pigment formers.
    a. Monosaccharide carbohydrates alone fermented.
        1. *Propionobacterium frendeureichii.*
    aa. Monosaccharides and lactose fermented.
        2. *Propionobacterium shermanii.*
    aaa. Mono and disaccharides, arabinose and xylose fermented.
        3. *Propionobacterium pentosaceum.*
    aaaa. Mono- di- and polysaccharides fermented.
        4. *Propionobacterium technicum.*
2. Pigment formers.
    a. Orange pigment.
        b. Mono- and disaccharides fermented.
        5. *Propionobacterium jensenii.*
        bb. Mono- and disaccharides and raffinose fermented.
        6. *Propionobacterium petersonii.*
        aa. Red pigment.

b. Raffinose and mannitol fermented.
c. Ratio of propionic acid to acetic acid 5:1
7. *Propionobacterium thonii.*
cc. Ratio of propionic acid to acetic acid 3:1
8. *Prionobacterium rubrum.*

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Section II

THE PRODUCTION OF ALCOHOLS AND KETONES

CHAPTER 8

Amyl Alcohol Production

Amyl alcohol is a recognized by-product of the alcoholic fermentation of sugars by yeasts. On distillation of the fermented molasses "beer" in industrial alcohol plants the "heads" or light fraction coming off first contains aldehydes which are usually discarded. The "tails" or high boiling heavy fractions are largely fusel oil, a mixture of higher alcohols, etc., principally amyl. Until recently this has been the principal source of amyl alcohol, although now it is being made synthetically from pentane.

Several of the earlier British patents (1, 2) based on the use of organisms of the acetobutylicum type claim to form amyl alcohol along with other higher alcohols and acids but probably any amyl alcohol formed was in reality a small unimportant by-product of the butyl fermentation.

In 1914 a United States patent was granted to Schenkenbach (4) for a "Process of Manufacturing Fusel Oil" by fermentation of carbohydrates with an unidentified spore-forming bacillus resisting 100°C. for half an hour. The process is not described in detail here for if carried out as directed either the *Clostridium acetobutylicum* or *Bacillus acetoethylicum* would probably be obtained and both of these fermentations have been discussed in detail.

There has been considerable difference of opinion as to the origin of the amyl alcohol in yeast fermentations, some believing that the higher alcohols are formed only when the yeast cells reach a stage of exhaustion towards the end of the fermentation, others that they are derived from the fat stored in the yeast cell and others that they are produced by associated bacteria. As no industrial fermentation practiced at present produces primarily amyl alcohol the discussion is not gone into further here. Lafar devotes considerable space to several species of bacteria known to be able to produce small amounts of amyl alcohol (3).

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CHAPTER 9

Butyl Alcohol and Acetone Fermentation

HISTORICAL

The butyl alcohol-acetone fermentation is one of the most interesting of the industrial fermentations and of recent years one of the most important and most carefully studied. The processes are covered by patents in Germany, England, the United States and other countries. We have listed 21 United States patents and 16 British patents but have made no effort to make the latter list complete or to list patents in other countries.

The first British (18) and American (19, 20, 21) patents issued in 1912 employed the Butyl bacillus of Fitz (1–6), but Fitz himself in his early reports on this organism did not recognize acetone among its fermentation products. The first German patent issued in 1913 (22) called for the use of *Bacillus mascerans* Shardinger. In 1914 a German patent called for the use of *Bacillus butylicus* Fitz. A British patent (24) employed a heat-resistant rod isolated from cereal grains or soil, growing either aerobically or anaerobically, for the production of acetone and butyl alcohol from a carbohydrate, as maize. The method of isolation did not insure a pure culture but the predominating organism was designated as *Granulobacter pectinovorum*. But little attention was paid to the process until the world war demand for acetone in the munitions industry resulted in its development to quite a large capacity both in England and Canada.

Starting as a war industry primarily for the production of acetone, with butyl alcohol as a by-product or a "white elephant" according to Gabrial (82), it has continued and developed primarily to supply butyl alcohol as an industrial solvent of wide usefulness and as a starting point for the manufacture of butyl acetate or propionate and various derivatives used in manufacture of perfumes. Acetone is now a secondary consideration.

At present the industry, in this country at least, is completely covered by patents, all or almost all of which are held by one company.

ORGANISMS USED

Early descriptions of the organisms used are incomplete and apparently the same organism has been given a number of different names by different workers and in different patent claims. The more recent American claims designate it as *Clostridium acetobutylicum.* Bergey's *Manual of Determinative Bacteriology* (89) groups 25 different synonyms together under the heading *Clostridium butyricum* Prazmowski, the original description of which agrees very well with those given in various patent claims with the exception of gelatin liquefaction (the organisms used today liquefying gelatin and being definitely proteolytic) and the presence of granules along the rod margins. The latter error is explained by the recognition by later workers of the fact that organisms with laterally located volutin granules were associated contaminations of the so-called *Bacillus volutans*, possibly identical with *Spirillum volutans* Ehrenberg, of the *Manual* (45). Weizmann and Hamlyn (37) state that they have used successfully six different organisms for butyl alcohol production, but all six are classed as the same organism by Bergey.

The organisms grouped under one heading in Bergey's *Manual* are as follows:

1. *Clostridium butyricum* Prazmowski
2. *Vibrion butyrique* Pasteur
3. *Bacillus amylobacter* v. Tieghem
4. *Bacterium navicula* Reinke u. Berthold
5. *Bacillus butylicus* Fitz (4)
6. *Butylbacillus E.* Buchner (16)
7. *Bacillus amylobacter I* Gruber (7)
8. *Bacillus amylobacter II* Gruber
9. *Bacillus amylocyme* Perdix (8)
10. *Bacillus butyricus* Botkin (9)
11. *Granulobacter saccharobutyricum* Beijerinck
12. *Bacillus orthobutylicus* Grimbert (10)
13. *Amylobacter butylicus* Duclaux (11)
14. *Granulobacter butylicum* Beijerinck
15. *Bacillus saccharobutyricus* vKecki
16. Motile Buttersaure bacillus, Grassberger u. Schattenfroh
17. *Clostridium der Heufroste* Behrens
18. *Clostridium pastorianum* Winogradsky (12)
19. *Plectridium plectinovorum* Stormer
20. *Clostridium giganteum* Kentner
21. *Clostridium americanum* Pringsheim, (14)
22. *Granulobacter pectinovorum* Beijerinck

23. *Granulobacter urocephalum* Beijerinck
24. Alcohol bildenes clostridium of Shardinger (15)
25. *Bacillus amylobacter* Bredemann (17)

Bredemann (17) as a result of an intensive study of a large number of organisms producing butyric acid fermentations came to the conclusion that numbers 7, 14, 15, 18, 21, 22, and 25 of Bergey's list and four others were certainly the same species and that numbers 1, 6, 8, 9, and 16 others listed by him were probably also of the same species. Among those included in the various patent claims as producing butyl alcohol and acetone the principal differences are in their relation to oxygen and their ability to liquefy gelatin. Bredemann does not think that variations in the latter function justify claims for species differentiation and a careful study of patent claims indicates that all of the organisms used are facultative and adaptable to the presence of free oxygen.

If these are not all the same organisms, usually incompletely described in the original manuscripts, they are certainly closely related and possibly varieties of the same species. Those used by Weizmann and Hamlyn were numbers 5, 11, 16, 18, 21, and 25 of the above list.

Commercial fermentations and patent claims include *Bacillus mascerans*, an aerobic organism of a different species, an Amylobacter, *B. butylicus*, *Granulobacter pectinovorum* and *Clostridium acetobutylicum.*

Weyer and Rettger (78) recently reported a complete study of six different strains of the organisms commonly used in large scale productions of butyl alcohol and acetone. They found no marked differences in physiology, all being powerfully diastatic, saccharolytic and proteolytic. The most complete description of the organism to be found is that given by the above authors and it is here quoted in considerable detail.

Vegetative cells in 5 per cent corn mash at 37°C. for twenty-four hours, short rods, single, 2 to 5 by 0.4 to 0.6 microns, rounded ends: Capsules in old glucose cultures.

Sporangia in 5 per cent corn mash after forty-eight hours at 37°C., spindle shaped, 4 to 6 by 1 to 1.2 microns. Endospores subpolar, cylindrical to elliptical 1 to 1.6 by 0.8 microns wall thick, non-adherent.

Motile with peritrichous flagella.

Irregular cuneate forms present in old glucose cultures.

Stains deep blue with methylene blue. Gram positive.

Granulose with Lugol's iodine (glycogen storage).

No growth on aerobic agar stroke.

In gelatin stab a slow limited growth, plumose in depth, slight liquefaction.

Malt extract gelatin agar, polyhedral granular colonies, 2 mm. entire, in five days; cream white viscid. Gas produced.

Broth—No growth.

Potato (anaerobic)—Slimy, opaque, cream colored flat growth, butyl odor.

Peptone starch, rapid and complete diastatic action.

Litmus milk—Rapid coagulation, peptonization, acid formation final reduction.

Best medium of growth 5 per cent corn mash.

Best medium for colony formation—Malt extract gelatin agar.

Quick differential test—Diastatic action and odor.

Non-pathogenic to rabbits and guinea pigs.

Viable for years in culture media.

Great tolerance to acids but slight tolerance to alkalies.

Very resistant to drying.

The malt extract gelatin agar used was as follows:

Malt extract	5 grams
Agar	20 grams
Gelatin	10 grams
Saturated solution $CaCO_3$	1000 cc.

Autoclaved, filtered, tubed and sterilized.

According to Bergey's *Manual*, indol (89) is not formed but nitrates are reduced. Acids, principally butyric and acetic, gas and alcohols are formed on sugar. The optimum growth range is 30° to 40°C. Weizmann (24) states that spores resist 90° to 100°C. for one to two minutes. Weyer and Rettger, (78) studying the effect of germicides on the organism, found it to be comparatively susceptible to such agents, with the exception of butyl resorcinal. Its resistance to this substance they attributed to the naturally high resistance of the solvents-producing species to the butyl radical which is responsible for the high toxicity of this compound toward other organisms generally. Weyer (86) has patented the use of butyl resorcinal in a 1:50,000 concentration added to the mash to lessen the necessary time of sterilization and to prevent subsequent contaminations.

ACTION OF THE ORGANISM

This organism will ferment not only hexoses but pentoses, disaccharides, polysaccharides and starches. Glucose, fructose, mannose, sucrose, lactose and starch are completely fermented with low acid production, galactose, xylose, arabinose, raffinose, melezitose, inulin and mannitol are completely fermented with high acid production and

dextrin is incompletely fermented with partial decomposition of the product. Trehalose, rhamnose, melibiose and glycerol are not acted upon. In carbohydrate mixtures the hexoses are completely removed before sucrose or lactose are acted upon (52).

Fermentation appears to be carried out in two stages. According to Speakman (38) the organism secretes an enzyme or enzymes which

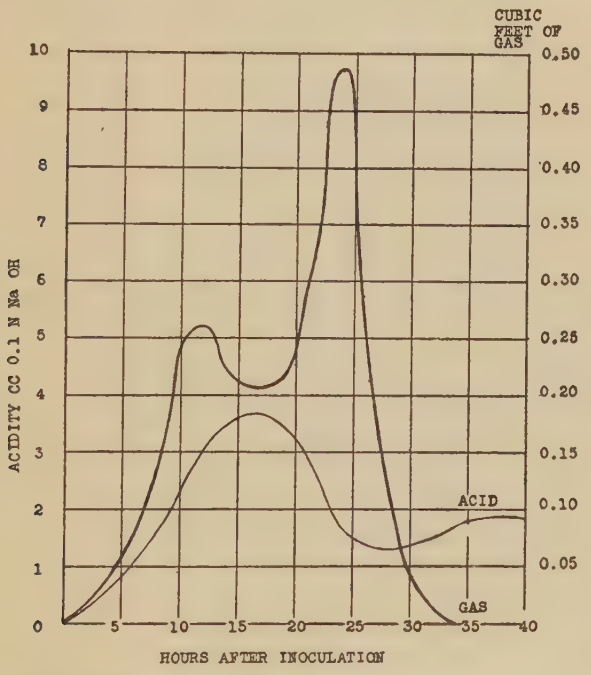

FIG. 5. CURVES SHOWING ACID AND GAS PRODUCTION DURING NORMAL BUTYL ALCOHOL-ACETONE FERMENTATION

(After Speakman)

hydrolyze starch to glucose. Glucose passes into the cell and is oxidized to butyric acid and acetic acid. Reilly (42) states that the ratio of butyric to acetic acid is highest with the highest total acidity and it then steadily decreases until the acetic acid exceeds the butyric. The addition of aceto-acetic ester will increase the acetone yield. In complete fermentation the acidity rises for a time, then the acids flow back into the cell and are converted to the neutral substances, butyl alcohol and acetone respectively, by reduction. In incomplete fer-

mentations the acidity remains high and little alcohol and acetone are formed.

Various formulae have been suggested to indicate the chemical action. Frieberg (61) suggests the following:

$$C_6H_{12}O_6 = \underset{\text{butyric acid}}{CH_3\cdot CH_2\cdot CH_2\cdot COOH} + 2H_2 + 2CO_2$$

$$C_6H_{12}O_6 = CH_3\cdot CH_2\cdot CH_2\cdot COOH + 2H_2O + CO_2 \text{ (C being used by cells)}$$

$$C_6H_{12}O_6 + H_2O = \underset{\text{acetic acid}}{2CH_3COOH} + 3H_2 + CO_2 \text{ (C and O being used by cells)}$$

$$C_6H_{12}O_6 = 3CH_3COOH$$

$$C_6H_{12}O_6 = \underset{\text{butyl alcohol}}{CH_3\cdot CH_2\cdot CH_2\cdot CH_2\cdot OH} + 2CO_2 + H_2O$$

$$C_6H_{12}O_6 + H_2O = \underset{\text{acetone}}{CH_3CO\cdot CH_3} + 3CO_2 + 4H_2$$

The accompanying graph (fig. 5) taken from Speakman (39) shows the changes in acidity and rate of gas production as fermentation progresses. He suggests the following formulae:

$$\underset{\text{starch}}{C_6H_{10}O_5} + H_2O = C_6H_{12}O_6$$

$$C_6H_{12}O_6 = \underset{\text{n-butyric acid}}{C_3H_7COOH} + \underset{\text{acetic acid}}{CH_3COOH} \ (O_2 \text{ used by cells}$$

$$2C_3H_7COOH + O_2 = \underset{\text{aceto-acetic acid}}{2CH_3\cdot CO\cdot CH_2\cdot COOH} + H_2$$

$$CH_3CO\cdot CH_2COOH = \underset{\text{acetone}}{CH_3COCH_3} + CO_2$$

$$C_3H_7COOH + 2H_2 = \underset{\text{butyl alcohol}}{CH_3CH_2CH_2CH_2OH} + H_2O$$

$$CH_3COOH + 2H_2 = \underset{\text{ethyl alcohol}}{CH_3CH_2OH} + H_2O$$

In this scheme the butyric acid is oxidized to aceto-acetic acid from which both acetone and butyl alcohol are formed, the latter by reduction with hydrogen. Ethyl alcohol is also formed by reduction of the acetic acid. These formulae account for all of the principle products of fermentation but the intermediate aceto-acetic acid has not been identified.

In actual fermentation these formulae may represent simultaneous or consecutive changes or they may represent merely end results through intermediate stages not recognized.

The organism is definitely proteolytic, producing hydrolysis of

native proteins in cultures, from 15 to 60 per cent of the nitrogenous material being rendered soluble (71). The presence of protein or complex amino acids in cultures is essential for satisfactory growth (78), the best ratio of carbohydrate to protein being 5.5:1. A lower ratio favors high acetone production with less alcohol and a higher ratio yields less acetone and more alcohol. According to Peterson, Fred and Domogalla (59) in a fermentation of corn mash this organism hydrolyzes the protein rapidly. From 50 to 75 per cent of the protein goes to soluble products in from three to four days, simple peptides and amino acids being formed. Owing to these buffers and acids of low dissociation a high titratable acidity may be produced without materially altering the pH value.

Wilson and Fred (87) recently stated that the organism cannot use ammonium salts as the sole source of nitrogen but that these are preferred in the presence of protein nitrogen. Up to 75 to 100 mg. per liter of ammonium nitrogen as chloride, sulphate or phosphate may be present without decreasing the yields of total solvents but the ethyl alcohol will be increased at the expense of the acetone. If more ammonium chloride is supplied acetone yields are increased but total solvents are decreased. With the use of ammonium carbonate there may be up to 175 mg. per liter of ammonium nitrogen. These effects are partly specific and partly due to altered pH values from the acid set free when ammonium nitrogen is used.

In normal fermentation two parts of butyl alcohol are produced for approximately one part of acetone. Fermentation is associated with the production of large volumes of gas consisting on the average of one volume of hydrogen to two volumes of carbon dioxide (45). According to Speakman (39) a small amount of pure hydrogen is produced at first and then the rate of gas production gradually increases while the percentage of hydrogen falls until the above ratio is obtained. In commercial fermenations these gases must be separated from the solvent vapors which are condensed. In one factory these gases are utilized for the production of synthetic methanol and ammonia, both processes being patented (81). Edmonds (88) describes an apparatus permitting the recovery and utilization of the gases in pure form.

END PRODUCTS OF FERMENTATION

Normal fermentation yields approximately 60 per cent of butyl alcohol, 30 per cent of acetone and 10 per cent ethyl alcohol (80) but

there are small amounts of other substances present. Folpmers (44) states that normal butyl alcohol, isobutyl alcohol, normal propyl alcohol, isopropyl alcohol, ethyl alcohol and their corresponding acids are formed. After distillation of the fermented mash there remains a dark amber high boiling residue having an odor of butyl alcohol and representing 0.5 to 1 per cent of the solvent yield. According to Marvel and Broderick (69) this contains butyl alcohol, active amyl alcohol, iso-amyl alcohol, hexyl alcohol and the esters of butyric, caprylic and capric acids. According to Peterson, Fred and Schmidt (57) the fermentations of the pentoses xylose and arbinose by *Bacillus granulobacter pectinovorum* give the same products in essentially the same proportions as does that of glucose. There are slightly less of solvents and slightly more of the volatile acids and the rate of fermentation is slower, but almost all of the sugar is destroyed in seventy-two hours. Wilson, Peterson and Fred (79) have recognized acetyl-methyl-carbinol as a regular product of the fermentation to the amount of 300 to 400 mgm. per liter. It is formed at the same time as the acetic and butyric acids but is more sensitive to modifying factors than are the solvents. It is increased by increase in phosphates and decreased by decrease in proteins. Yeasts can also produce acetyl-methyl-carbinol, forming it from acetaldehyde.

$$CH_3CHO + HOC{\cdot}CH_3 = CH_3CHOHCOCH_3$$

Schmidt, Peterson and Fred (58) have identified a non-volatile acid formed in this fermentation as *a*-hydroxy-iso-caproic acid or *b*-leucic acid, the amount formed increasing with the age of the culture.

ISOLATION OF THE ORGANISMS

Weizmann (29) in his first American patent describes the method of isolation of the organism from natural sources. As adapted by Weyler and Rettger (78) the method is as follows:

To a number of test tubes each containing 10 cc. of sterile 5 per cent corn meal mash were added small amounts of the unsterilized materials in which the organism is being sought. The tubes are then immersed in boiling water for forty-five seconds and immediately cooled in running water. They are then incubated for twenty-four to thirty-six hours and examined for odor and gas production. All not showing vigorous amylolytic action and definite butyl odor are discarded. The remainder are further incubated until action ceases or for eight to

ten days. They are then reheated in boiling water for forty-five seconds, cooled and the contents plated on malt gelatin agar in varying dilutions, using Krumweide and Pratt's (23) inverted plate method. After inoculation of the liquefied malt gelatin agar the medium is poured into the inverted lid of a sterile petri dish and the bottom section of the dish then floated on the liquid. After incubation the two parts of the plate are easily separated and the colonies fished. Within two days typical polyhedral colonies, when present are fished into fresh tubes of sterile 5 per cent corn mash. While most workers prefer to keep their stock cultures in sealed tubes in 5 per cent corn mash, Freiberg (60) keeps his stock cultures on potato stabs.

SOURCE OF ORGANISMS

Using similar methods organisms of this type have been isolated by various workers from fresh cow manure (3), soil, corn meal, barley meal, putrefying clams (78), market milk (9), slaughter house refuse (24), and other sources. We ourselves have isolated it from corn meal, potatoes and market milk, the latter accidentally.

FERMENTABLE RAW MATERIAL

Various substances have been suggested and used as raw materials for butyl alcohol and acetone production. Most of the commercial work has used corn meal but patent claims include the use of cereal grains, rice, wheat, oats, rye, durra, or potatoes (24), molasses (62), beets (64), horse chestnuts (31), chestnuts (50), hydrolyzed woody matter (18), and in general terms any fermentable carbohydrate. When horse chestnuts are used the saponin present in the husks must be removed by preliminary washing (31) and the yields are less than from corn. Robinson (56) uses a low grade molasses, decolorized with activated charcoal to remove inhibiting toxins. Among other substances used or recommended commercially are artichokes (77) and jawari flour in India (48).

COMMERCIAL SCALE FERMENTATIONS

In commercial fermentation in large bulk it was first thought advisable to have the vats contain some inert material (35) unless the raw material contains such. One process used twigs and sticks and another suggested the use of asbestos, filter paper, peat, fruit peels or potatoes in the mash but this does not seem necessary, at

least with cereal meal which contains more or less fiber. This material also contains essential nitrogenous matter. Where pure carbohydrates are to be acted upon nitrogenous material must be added for bacterial nourishment. Autoclaved yeast (26) has been so used. It furnishes albuminous substances and inorganic nutrient salts. Fernbach and Strange (19) used yeast degraded by inoculation with *Tyrothrix tenuis*.

In plant practice the organism is cultivated in the 10 cc. tubes of sterile corn meal which are incubated until the amylolytic fermentation ceases as indicated by subsidence of the flaky scum and separation of a watery surface layer. These tubes may then be used to seed 3-gallon mashes which when actively fermenting are in turn used to seed 800 gallon tanks which after sixteen hours fermentation are used to seed tanks of 3000 gallons or more (33). These amounts of course are subject to variations but the principle of successive increase in volume of seeding batches is always followed, though each successive fermentation does not start from a small seeding tube. For storage the seeding tubes are sealed in the flame after complete fermentation and when so stored the spores have remained viable for seven years (68). However after six months storage they are less active and will not give good yields unless rejuvenated by a series of subcultures (78).

MAINTAINING ANAEROBIOSIS

Fernbach's patent (19) called for the production of an inert gas as carbon dioxide or nitrogen, over the cooling mash after sterilization so that action would be entirely anaerobic. After fermentation is well established there is sufficient evolution of carbon dioxide and hydrogen in the closed vats to displace all air and insure anaerobiosis. For the purpose of isolation and seeding tube preparation it is advisable to secure anaerobic action by one of the recognized methods. Weyer and Rettger (78) found that very good results were obtained by inoculating the sterile tubes of corn mash with a *Staphylococcus aureus* culture and incubating for twenty-four hours before inoculating with the clostridium or the material from which isolation is to be attempted. The *S. aureus* absorbs oxygen from the mash, insuring anaerobiosis, and is itself killed off in twenty-four hours or a little longer by the clostridium or its metabolic products so that the fermented mash is safe to handle. We have ourselves proved this to be a satisfactory procedure.

PREDOMINATING CULTURE FERMENTATIONS

In most commercial and experimental work extreme care has been taken to insure pure culture fermentations and in large factory installations this is often very hard to do. That this may not be necessary except for first batches however is suggested in the American patent of Ricard (64) in which to a large volume of actively fermenting and previously sterilized wort small amounts of unsterilized wort are added at hourly intervals after the withdrawal of similar amounts of fermenting wort, which are then fermented to completion in separate containers. They state that this process may be repeated 50 to 60 times. Evidently the establishment of a predominating actively fermenting culture is usually sufficient to insure lack of interference from ordinary contaminants.

CONTAMINATIONS

Thaysen (45) describes a *Bacterium volutans* as the most dangerous common contaminant. Its physical and morphological characteristics resemble the acetobutylicum or amylobacter as Thaysen calls it. It produces lactic, acetic and butyric acids from sugars but does not attack starch. It may be distinguished by its laterally located volutin granules stained deep purple by methylene blue and dark by Lugol's solution. The granules in the acetobutylicum are terminal. Fred, Peterson and Carroll (67) describe a red-pigment-producing contaminant forming acetone and ethanol which they provisionally identify as *Bacillus mesentericus ruber*.

In a discussion of the effect of lactic-acid-forming bacteria on fermentation by the "*Granulobacter pectinovorum*" Fred, Peterson and Mulvania (74) state that the presence of the granulobacter is an aid to lactic acid fermentation but that all of the lactogens inhibit the solvent fermentation to some extent. *Lactobacillus leichmanni* is most harmful, the mannitol fermenters somewhat less so and *Lactobacillus intermedium* and the *Streptococcus lactis* least. These lactogens may persist a year or more in the corn mash with the granulobacter, being favored by vigorous fermentations with the formation of buffer substances. The harm done by these lactogens is not specific, as acidity of any type to a pH value from 4.7 to 4.8 always has an inhibitory effect on the acetobutylicum.

CAUSES OF POOR YIELDS; EFFECT OF PASTEURIZATION

Failure to obtain good yields of solvents is not always due to the presence of contaminating organisms. It has been demonstrated that after repeated transfers of cultures containing many vegetative forms and few spores the predominating vegetative cells seem to weaken in their enzyme production (78). Much more constant high yields are obtained if repeated transfers for seeding batch preparation are interrupted periodically and the weak fermenters removed by pasteurization, as this removes vegetative forms that have lessened in enzyme production and also the weaker, less-resistant, of the spores. Old cultures that have lost their ability to give good yields may be rejuvenated by successive pasteurizations of subcultures, as also may spore tube cultures that have been stored too long in the dormant stage. Six months should be the limit of storage of spore tubes to obtain good yields. At least two of the American patents recognize the value of pasteurization in stimulating solvent production (62), (84). Freiberg concluded that the excess of calcium and magnesium salts in hard water interfered with the activity of the amylobacter and in one of his patents (85) he claims to increase solvent yields by softening the water used in preparing the mash.

ASSOCIATED ORGANISMS

While the *Clostridium acetobutylicum* is the organism of choice for butyl alcohol and acetone production, other organisms may produce similar results or be an aid to production. Strange (65) has employed yeasts as well as bacteria and Weizmann and Hamlyn (37) inoculate their maize or other material with a strongly proteolytic fungus, *Aspergillus oryzae*, and hold at 37°C. for four days before inoculation with the amylobacter. At the end of this period the mash is inoculated with the amylobacter without re-sterilization. The patent claim states that an enzyme prepared from mold may be used instead of the mold itself.

TEMPERATURE OF FERMENTATION

While commercial fermentations are usually conducted at a temperature of 37°C. or over, Freiberg (66) finds that when solvent yields fall off a lowering of the temperature to from 30° to 35°C. may restore active butyl fermentation.

RELATIVE SOLVENT YIELDS

Various workers have noted that the relative amounts of the two principle solvents formed may be materially influenced by the addition of certain chemical substances to the fermenting mash. Speakman (38) states that the addition of butyric acid increases the yield of butanol and he also states that if propionic acid is added propanol is produced therefrom. Reilly and Hickenbottom (42) state that the periodic addition of acetic acid will increase acetone yield, it being almost wholly converted into acetone. They also state that in the presence of calcium carbonate the acids are formed but no alcohols or acetone. Desborough (30) states that volatile fatty acids or their salts may be added to the mash to increase or modify the yield. Lactates have also been suggested as additions to the wort from beet roots to increase butyl alcohol formation (54). The effect of proteins has already been discussed.

When a series of fermentations is run, the relative content of solvents of each may be quite accurately gauged by the color of the fermented liquid. The butyl alcohol formed dissolves some of the zein from the corn imparting a golden yellow color to the liquid (78). Depth of color depends on amount of dissolved oil and this depends on concentration of butanol.

USE OF RESIDUE

Killeffer (80) states that after the fermented mash has been freed from formed solvents the residue may be used for fertilizer. Fowler, Wade and Gokhali (48) state that after fermentation of jawri flour in India, a cake utilizable as cattle food is obtained by pressing out the sludge and the turbid supernatant liquid when clarified, with milk of lime, and filtered or allowed to settle out gives a good fertilizer cake.

From what has been given above it may well be realized that as Speakman states (40) the obtaining of a maximum yield of solvents in a minimum time depends upon correct volume proportions between seed cultures and mash, the stage at which culture should be withdrawn for new seed, the number of generations between subculture and fermentation and other variables.

AVERAGE YIELDS

Chemical methods of estimating yields of acetone and butyl alcohol are given in the paper of Weyer and Rettger (78). An idea of the

yields to be expected may be obtained from the following figures quoted from various sources.

Thaysen states (45) that 100 parts of starch give 12 parts acetone and 24 parts butanol.

Weizmann and Hamlyn, using *Aspergillus oryzae* (37) followed by amylobacter obtained a 15 to 20 per cent yield of mixed products.

In another article Thaysen (75) states that starch is convertible into 10.77 per cent acetone, 25.07 per cent butanol, 62.61 per cent carbon dioxide, 1.6 per cent hydrogen and 1.8 per cent organic acids.

Fowler and others, in India, (48) from 1200 grams of jawari flour obtained 70 cc. of acetone and 128 cc. of butanol.

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CHAPTER 10

Butyl Alcohol and Isopropyl Alcohol Production

At the annual meeting of the Society of American Bacteriologists, held in Philadelphia in 1927, Prescott and Moriskawa (1) presented a paper on the production of butyl and isopropyl alcohols from carbohydrates by fermentation with an organism isolated and named by them *Bacillus technis*. This paper as abstracted gives no details of organism or method, but Moriskawa published (2) in a Japanese journal a detailed account of both.

The organism was isolated from Kojii mash in Japan. It is described as follows:

> Rods, 2 to 5 microns by 0.7 to 1.0 microns, granular in old cultures, spores central or excentric 1.8 to 2.5 by 0.6 to 1.25 microns dilating rods. Many bizarre forms in old cultures. Motile when young. Agar slant—filiform abundant, echinulate, raised, glistening, opaque, butryous. Agar colonies—circular, irregular margins, smooth or rough, flat, finely curled, gelatin stab rapid and complete liquefaction. Potato—abundant, dirty white becoming brownish, butyrous. Broth—turbid with abundant compact sediment. Milk—acid, coagulation, gas, some slow digestion and slow litmus reduction. Starch digestion in corn meal mash. Gas production variable with different carbohydrates. No indol or hydrogen sulphide formed. Nitrates reduced to nitrites and ammonia. Aerobic, facultative. Optimum temperature 30° to 40°C. Minimum 10°C. Maximum 50°C. Optimum pH value 6 to 7. Positive methyl red.

Sugars fermented to yield chiefly *n*-butyl and isopropyl alcohols with variable ratios of hydrogen and carbon dioxide gas. Small amounts of acetone are formed under reduced oxygen tension. No ethyl alcohol is formed. This organism is not listed in Bergey's *Manual* but from the description it is closely related to *Clostridium acetobutylicum* (see table 4).

Aldehydes are formed as intermediate products. Early abundant gassing, hydrogen gas predominating. Later $H : CO_2$ ratio is 4 or 5 to 6 or 4. Acidity reactions similar to those with the butyl alcohol-acetone fermentation. Growth is inhibited by too high acidity, excessive sugar concentration or the presence of too much alcohol at start of fermentation. In Koji mash fermentations the pH value may be-

come 4.6. In dextrose broth fermentations are arrested by a pH value of 5.0. Normally fermentation proceeds to a pH value of from 4.4 to 4.2, with a titratable acidity of 3 cc. N/10 sodium hydroxide per 10 cc. of mash. Fermentation is inhibited by the presence of lactic acid. A 12 per cent sugar medium (glucose) gives the highest yield of alcohols. One hundred grams of sugar yields 30 grams of alcohol

TABLE 4

FERMENTATION OF CARBOHYDRATES

SUGAR	GAS	REACTION
Rhamnose	Little	Alkaline late
Arabinose	Much	Acid
Xylose	Much	Acid
Mono-saccharides	Much	Acid
Di-saccharides	Much	Acid
Adonite	Little	Alkaline late
Dulcite	Little	Alkaline late
Mannite	Little	Acid
Glycerin	Little	Acid

with a 3 to 1 or 2 to 1 ratio of the butyl to isopropyl alcohol. Fermentation is stimulated by the presence of calcium carbonate and ammonium hydrogen phosphates.

At the time of writing it is understood that this fermentation is about to be tested on a commercial basis, but no further details are available.

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CHAPTER 11

Ethyl Alcohol Production

The consideration of the details of the alcoholic fermentation will be confined to the production of industrial alcohol. While the preparation of the fermented alcoholic beverages may be properly classed as industrial yet it is a highly specialized industry with a large and comprehensive literature of its own. The proper control of flavor and aroma are essential and require detailed study not gone into by the average industrial biologist. Also as this industry is taboo in this country there should be little need for discussion of its problems.

Over one hundred years ago (1815) Gay Lussac (1) recognized alcoholic fermentation with the production of two molecules of alcohol and two of CO_2 from one molecule of dextrose. $C_6H_{12}O_6 = 2CO_2 + 2CH_3CH_2OH$. Fermentation chemistry was not further clarified until 1912 when the surprisingly easy fermentation of pyruvic acid by yeast at room temperature was recognized.

$$CH_3COCOOH = CH_3COH + CO_2$$
Acetaldehyde

This action is due to the enzyme carboxylase (10). Then step by step the intermediate stages of the fermentation were worked out until we have the complicated series of reactions now recognized as at least a partial explanation of what occurs.

According to Nord (17) the following sequence of formulae express the fermentation reactions as understood today in the formation of alcohol from sugar.

1. Two molecules of sugar are acted on, one splitting directly into alcohol and CO_2. The second unites with a metallic phosphate to form hexose-diphosphate and water.

$$C_6H_{12}O_6 = C_2H_5OH + CO_2$$
$$C_6H_{12}O_6 + 2PO_4HM_2 = C_6H_{10}O_4(PO_4M_2) + 2H_2O$$

2. The hexose diphosphate continually breaks down to yield fermentable or labile hexose, due to the enzyme phosphatase. During the fermentation the phosphates act as buffers enabling the enzymes

to maintain more or less permanently a definite pH value at the place of enzyme activity within the cells (a pH value of 6).

The externally produced diphosphates penetrate into the cell until a suitable salt concentration is reached which brings about the coagulation of the cell membrane. Through the interstices of the now crystalline membrane the hexose enters the cell where it is esterified by the endocellular synthease. The hexose diphosphates are altered to a transportation form of the sugar.

$$C_6H_{10}O_4(PO_4M_2)_2 + 2H_2O = C_6H_{12}O_6 + 2PO_4HM_2$$

3. The fermentable hexose looses water to form methyl glyoxal aldol (theoretic)

$$C_6H_{12}O_6 - 2H_2O = C_6H_8O_4$$

4. Which splits into two 3-carbon-chain methyl glyoxals.

$$C_6H_8O_4 = 2CH_2{\cdot}C(OH)CHO \text{ or } 2CH_3COCOH$$

The changed salt concentration causes repeptization of the cell membrane completing the cycle of intermittent coagulation-peptization of the cell membrane.

5. Two molecules of methyl glyoxal unite with two of water to form glycerol and pyruvic acid.

$$\begin{array}{lll} CH_2C(OH)COH & H_2 + H_2O & CH_2OHCHOHCH_2OH \\ + & | & = \\ CH_2C(OH)COH & O & CH_2{:}C(OH){\cdot}COOH \end{array}$$

6. Pyruvic acid is an intermediate to acetaldehydes being split by the enzyme carboxylase.

$$CH_2{:}C(OH){\cdot}COOH = CH_3COH + CO_2$$

7. Two different aldehydes, methyl glyoxal and acetaldehyde, unite with water to form pyruvic acid and alcohol.

$$\begin{array}{lll} CH_3COCOH & O & CH_3COCOOH \\ & + \; | = & + \\ CH_3COH & H_2 & CH_3CH_2OH \end{array}$$

Thus there is an uninterrupted formation and decomposition of pyruvic acid, which cannot accumulate.

Neuberg and Leibowitz (14) in 1927, working with yeast extracts,

came to the conclusion however, that the mono and not the diphosphoric acid ester is the long sought obligate intermediate product of fermentation. This excludes the factor of cell permeability.

Zymases are in reality enzyme complexes, rather than simple enzymes. According to Waksman (13) the phosphate acts as a co-enzyme or accelerator for the zymase.

The yeast zymase is capable of acting on four of the sterioisomers of hexose, *d*-glucose, *d*-mannose, *d*-levulose and *d*-galactose. It will ferment only those sugars whose carbon atoms are devisable by three.

Though equations as given may be worked out for many enzyme actions it must be borne in mind that enzymes rarely behave in strict accordance with the various chemical fomulae, most reactions being very complex.

The simple reaction first given to represent start and ending of alcoholic fermentation is known as Neuberg's first fermentation reaction. Neuberg's second and third reactions will be discussed in the chapter on glycerin production. The second occurs in the presence of sodium sulphite and the third in the presence of other alkaline salts as follows:

$$2\ C_6H_{12}O_6 + H_2O = \underset{\text{Acetic acid}}{CH_3COOH} + \underset{\text{Alcohol}}{C_2H_5OH} + 2\ CO_2 + 2\ \underset{\text{Glycerin}}{C_3H_8O_3}$$

and occurs near the completion of the alcoholic fermentation.

Theoretically, with the first reaction 180 grams of sugar should yield 92 grams alcohol and 88 grams CO_2 but this is never obtained. A well conducted molasses fermentation however, should give well over 90 per cent of the theoretic 95.2, being about the maximum. Some of the sugar is consumed for cell nutrition and some to form higher alcohol and aldehyde by-products.

Among the non-volatile by-products are succinic acid and glycerin, the latter being increased by reduced pressure, increase in amount of inoculum and increase in temperature. It is to some extent a racial peculiarity of some yeasts but may also be increased by increase of nitrogenous food stuffs. It increases toward the end of fermentation.

Among volatile by-products are aldehydes and volatile acids, as formic, acetic, propionic, butyric, valeric, caproic, caprylic, pelargonic and capric. Also higher alcohols, especially iso-amyl and *d*-amyl, combining to form the crude fusel oil of commerce.

Swenarton (18) states that industrial molasses fermentation has "fusel oil" as a by-product, obtained by distilling with alkali. The fraction below 131°C. is commercial fusel oil, or impure amyl alcohol. That over 131°C. is a complex mixture of *n*-propyl and iso-butyl alcohols, secondary butyl and iso-butyl carbinol, and hexylic, heptylic, octylic, noylic and decylic alcohols, probably normal and two members of each series having the isomeric structure of amyl alcohols and the above carbinols. F. Ehrlich (2) states that the higher alcohols are formed in yeast fermentation from the amino acids, leucin and isoleucin.

Disaccharides and polysaccharides must first be transformed to monosaccharides. No yeasts are able to attack starch or cellulose. Most yeasts produce maltase which hydrolyzes maltose to two molecules of dextrose. Many will ferment sucrose and a few lactose.

For alcohol manufacture the cheapest most available carbohydrates are starch and cellulose, which may be hydrolyzed by malt, by heat and acid respectively. Mold diastase may also be used for starch (8). However, most of the industrial alcohol made at present in the United States is made from non-edible black strap or crude molasses. It may be made from any substance capable of undergoing alcoholic fermentation, the limiting factor of practice being, principally, the cost of the raw material per unit of alcohol (5).

While aeration increases the action and reproductive power of yeast, yet continued aeration, as practiced in growing yeasts to market, may remove volatile products and oxidize alcohols to aldehydes.

In fermenting mashes alcohol accumulation stops cell reproduction before it checks fermentation. Fermentation is checked earlier at higher temperatures. At 9°C. fermentation though slow may continue to 9.5 per cent alcohol with one yeast, at 18°C. the same yeast may only produce 8.8 per cent, at 27°C. only 7.5 per cent and at 36°C. only 3.8 per cent alcohol.

Yeasts, however, can be habituated to higher alcohol percentages and alcohol concentrations up to 12 per cent may be so obtained. During fermentation cells sediment as they become inactive and change to resting cells which will not ferment. It is best to always prepare fresh starter cultures from pure culture test-tube fermentations as when the process is made a continuous one by leaving in the vat a considerable portion of one fermentation as seed for the next, the

yeast in time becomes weak and badly contaminated and lower yields are obtained.

With molasses rich in nitrogen no accessory nitrogen need be added but with some molasses it is best to add nitrogen in the form of ammonium sulphate equivalent to 0.008 per cent of nitrogen. Nitrogen concentrations above this point decrease yeast activity.

Richards (11) in studying the effect of calcium sulphate on the growth and fermentation of yeast found that the best concentration of calcium sulphate for the most efficient growth and fermentation of *Saccharomyces cerevisiae* is about 0.0001 molar. Higher concentrations of the salt inhibit growth and fermentation and lower concentrations are inadequate for best results. A study of the calcium sulphate content of water supplies shows that the concentration is usually greater than the optimum and may occasionally be fifty times as great, requiring water softening to obtain good yields.

When cane molasses containing 50 to 60 per cent of fermentable sugars is used as raw material it is diluted 50 per cent with water (not too high in calcium sulphate) to facilitate pumping, nearly neutralized with lime if too acid (to a pH value 5.0 (3)), pasteurized with steam for thirty minutes to remove most of the contaminating bacteria and then further diluted with water to 15° to 18°B. pumped into fermenting tanks at 30°C. and seeded with actively fermenting twenty-four-hour "bub". Usually a fresh 24 hour test-tube culture, of 25 cc., is used to seed a 200 to 500-gallon batch. This serves for several days seedings of larger vats, 1000 gallons or over, which are in turn used to seed the final fermentation of 100,000 gallons or more. Seed cultures should always be examined under the microscope to insure richness in yeast cells and freedom from contamination.

The temperature of fermentation must be maintained by removing excess heat with brine tubes, or more simply by a constant spray of cold water trickling down the outside of the fermentation tank.

Active fermentation is continued until specific gravity ceases to fall, usually in about forty-eight hours. The fermented "beer" is then pumped to the still house where the aldehydes are driven off in the heads and the alcohol and fusel oils recovered. If the fermenters are covered the CO_2 which is evolved in large quantities may be recovered and compressed to a liquid or used for the manufacture of "dry ice."

Molasses from beet sugar is diluted and acidified as above, pasteur-

ized and boiled for fifteen minutes to drive off volatile acids and oxides of nitrogen which would prevent yeast fermentation, then it is cooled to 30°C. and inoculated (5).

Where starchy raw materials are used they may be saccharified by acid and heat hydrolysis, by barley malt or by mold diastase from Aspergillus or Mucor (see diastase chapter). Acid hydrolysis is also used to convert cellulose materials such as saw-dust, corn cobs, hulls of various kinds of vines, etc., to sugars. In the hydrolysis of cellulose the hexoses formed may be converted to alcohol by yeast fermentation as above and the beer then sterilized and inoculated with lactobacilli to produce fermentation acids (see chapter on xylose fermentation).

Where malt is used the processes followed in the brewing industry are employed except that germination is carried further and the malt used green to produce as complete conversion as possible in as short a time as possible. The barley grain is steeped in sterile pure water at 12.5°C. for seventy-two hours to germinate. The green undried malt is then mixed with water at 35° to 40°C. gradually raised with hot water to 60° to 65°C. Holding it at this temperature for two hours or more results in saccharification of the starch. It is then ready for boiling to check enzyme action, dilution or concentration to the proper sugar content and fermentation with yeast. Moderate development of lactic acid during the mashing period, due to the presence of lactobacilli, will be an aid in preventing development of harmful contaminations. Mold diastase is not much used in this country.

Where molasses is shipped in copper lined tanks it should be tested for copper before use and attempts made to remove excess copper. Lafar (4) states that 25 mgm. of Cu per liter will retard fermentation.

Mezzadroli (7) reports the production of alcohol from vegetable ivory, by first producing mannose by acid hydrolysis, using button factory waste.

Bettinger and Delavile are quoted in *Abstracts of Bacteriology* (6) to the effect that mucors are used in the Far East for alcohol production but no details are given and it may be that they are merely used to saccharify the starchy material preliminarily to yeast fermentation, as in the amolaces process used in Europe (4).

Though alcoholic beverage production is not discussed in detail brief consideration will be given to some differences in yeasts and yeast cultures for various uses (12).

Yeasts are selected for different purposes according to their peculiarities and are grown so as to encourage specific functions. The yeast grower seeks the most active cells while the beer brewer seeks the best and most end-products.

For pressed yeast rapid action is essential and cultivation is carried on in the most favorable medium with ample oxygen supply at 30°C. in dilute solutions. The small amount of alcohol formed is used by the cells or carried off in the circulating air. Continuous culture methods are employed and sugar concentration is maintained by feeding fresh carbohydrate sufficient to keep a Balling saccharometer at a fixed height.

Beer brewers may use either bottom or top yeasts. Bottom yeast has a low albumen and high fat content and is a weak fermenter. There is little or no flocculation and poor precipitation with difficult clarification. Over ripe yeasts, high in albumen and rapid fermenters must be regenerated in sugar solutions with nutrient salt but no nitrogen. The addition of much yeast produces poor fermentation with many old inactive cells present. The addition of little yeast makes for many young active cells if there is a free oxygen supply and early stirring.

Top yeast fermentations are used for special beers. Cultures are used for successive fermentations. Heavy seedings and free aeration are essential, also much motion, high sugar content and warmth. Distilleries use yeast educated to produce high alcohol yields, usually *S. cerevisiae.*

Wine yeasts may ferment to over 16 per cent alcohol if started in alcohol-free substrates. Pure cultures are used to reinforce the yeasts and bacteria naturally present on the fruit. Grape yeasts give special boquets and aromas due to volatile by-products.

Special lactose fermenting yeasts are used to ferment milk for Kefir and have been used for flavor in margarine factories.

During the World War a so-called mineral yeast food for man and animals was prepared in Germany. A poor fermenting Torula was cultured in a very dilute molasses (2.5–3 per cent Balling) with superphosphate, magnesium sulphate and ammonium sulphate with free aeration. No alcohol was formed and there was no evident fermentation but in eight hours 100 grams of molasses produced 130 grams of yeast.

Birchner and Paine (16) describe the experimental use of specially

selected invertase-free yeasts in the desugarization of final cane molasses. Seven out of 16 strains of *S. apiculatus* tested, produced no invertase and have been termed pseudosaccharomyces. They however, may develop invertase if their food supply is low. After culture at 25°C. for several generations on wort agar they are grown on molasses wort and inoculated into the molasses. The levulose and dextrose present are fermented to alcohol, which is distilled off and the sucrose then recovered as calcium sucrate. The residue is used for fertilizer.

Takamine (10) has suggested the use of taka-diastase in the preparation of an alcoholic ferment mash. Wheat or other cereal bran is mixed with from 3 to 10 per cent of its weight of takakoji or the diastase equivalent of takakoji-diastase and then added to 4 to 8 volumes of water, gradually heated to 65°C. for fifteen minutes then to the boiling point for twenty minutes. It is then cooled to 60°C., reinoculated with takakoji, strained and the clear liquid boiled. It is then cooled to about 15°C. and seeded with yeast and incubated twelve to sixty hours. Settling precipitates the yeast cells formed which are used as a paste or pressed as seed for brewing or as bread yeast.

Vasseaux (9) patented a process of producing alcohol by mixing ulmin, ulmic acids, humin, humic acids and their congeners with a liquid containing a fermentable carbohydrate. The object of the process is to substitute a nutrient cheaper than sugar for the growth of the microörganisms, as the Mucors, this leaving the sugar or other carbohydrate subject to the action of the elaborated enzyme. Grain or malt residues mixed with grain or bran, fruit dregs and refuse, wine press residues, vine branches, beet tops, potatoes, Jerusalem artichokes, chestnuts, beech nuts, flax sprays, bran, straw, tan residues, sawdust, seaweed and molasses residues are treated to produce humic substances by chemical means, or the substances may be already available in a natural state. The humus is thoroughly mixed with thirty to fifty parts of the carbohydrate to be fermented. The temperature is maintained at about 30°C. as it is filtered and then diluted with cold water to a density of about 1.040 and inoculated with a pure culture of yeast cells or mold spores.

Hildebrandt and Boyce (19) discuss the stimulating effect of minute amounts of certain metallic salts, as $MnSO_4$, sodium cyanide and $CnSO_4$, on the yeast fermentation of cane molasses. They suggest 1–5000 $MnSO_4$, 1–2000 $CnSO_4$, 1–12000 NaCN. The stimulating

effect of these salts used in the growth of seed cultures was carried over into the larger fermentations thus minimizing the amounts of salts necessary to use.

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CHAPTER 12

Glycerin Production

Glycerin has long been recognized as one of the by-products of the alcoholic fermentation of yeasts as pointed out by Pasteur (1), and as early as 1916 we find a United States Patent (3) for the recovery of glycerin from distillery slops, but with no special effort made to increase the glycerin yields. Though it was recognized as mentioned in the chapter on ethyl alcohol production, that it was possible to increase glycerin yields to some extent by addition of select yeast, Pasteur (1) was unable to find more than 3 per cent of the fermented sugar as glycerin.

NEUBERG FERMENTATION REACTIONS

Neuberg (7) in his classic fermentation studies showed the influence of alkali and sodium sulphite on the fermentation. His first fermentation reaction, the simple conversion of hexose into alcohol and carbon dioxide occurred in the presence of ample air supply and under acid conditions. His second reaction type occurred in the presence of sodium sulphite and with limited air supply. As written by Neuberg (7).

$$C_6H_{12}O_6 = CH_3CHO + CO_2 + C_3H_8O_3$$

One molecule of hexose forms one molecule of acetaldehyde, one of carbon dioxide and one of glycerin, but this does not explain the function of the sulphite. As given by Waksman (11) the sulphite forms an aldehyde sulphite addition compound blocking further alcoholic fermentation steps. This compound concentrates in the mash and can be separated. The molecule of water contributes one hydrogen atom to the addition compound and the hydrogen which cannot act upon the acetaldehyde acts upon the other half molecule of sugar to form glycerin and sodium bicarbonate.

$$C_6H_{12}O_6 + Na_2SO_3 + H_2O = CH_3CHO \cdot H \cdot O \cdot SO_2 \cdot Na + NaHCO_3 + C_3H_8O_3$$

The ratio between glycerin and aldehyde is constant at any given time. The blocking of the acetaldehyde can be produced by absorption on animal charcoal.

In the presence of simple alkali salts the acetaldehyde is replaced by acetic acid and alcohol with two molecules of glycerin for one of acetic acid and we have Neuberg's third fermentation reaction.

$$2C_6H_{12}O_6 + H_2O = C_2H_5OH + CH_3COOH + 2CO_2 + 2C_3H_8O_3$$

This reaction takes place to some extent toward the end of the alcoholic fermentation accounting for the by-product glycerin found. In general the fermentation is a tool of physiologic molecular reduction to form acetaldehyde. This changes when substances uniting with nascent acetaldehydes are present.

INDUSTRIAL DEMAND A WAR NEED

The demand for glycerin during the world war increased interest in these fermentations and resulted in the establishment of fermentation factories where glycerin was the main product rather than a by-product. In this country a patent was granted in 1918 to Eoff (4) for the manufacture of glycerin using an alkaline medium resulting in Neuberg's third fermentation reaction. A patent for recovery of glycerin from fermentations granted in 1922 makes specific references to this method (8). Recovery is based on precipitating the fermented mash with a mineral acid removing thereby a large portion of the organic solid materials, concentrating the liquor by heat to a solid mass and bleaching with a solvent, as water or alcohol, and steam distilling the glycerin into an air-cooled condenser, the water vapor passing into a second water jacketed condenser.

ALKALINE PROCESS

Ling (5) describes the alkaline fermentation process as conducted on a semi-plant scale. In his work *Saccharomyces ellipsoideus* (Sternberg No. 657) was employed. Sodium carbonate was added in small amounts periodically, sufficient to form a precipitate and check gas evolution. This was started as soon as fermentation became active. The yeast was invigorated by seeding from a preliminary "bub" containing ammonium chloride in 17.5 to 20 per cent sugar. From 20 to 25 per cent of the sugar used was converted to glycerin and the rest to alcohol and carbon dioxide. Describing a black strap molasses fermentation he proceeds as follows:

150 cc. of sterile grape juice was seeded with yeast and fermented to quiescence.

15 cc. of this was used to seed 150 cc. of sterile grape juice which is again fermented to quiescence.

75 cc. of the second fermentation sufficed to seed 800 cc. of sterile black strap diluted to a specific gravity of 1.085. As soon as this is fermenting briskly 3 grams of soda ash are added. When again fermenting he added 2 gallons of black strap and when this showed active gassing repeated the soda ash addition. After another fermentation he added to 40 gallons of dilute molasses containing ½ pound of ammonium chloride. When active he added 2 pounds of soda ash and on development of fresh active fermentation added to the main mash of 355 gallons of dilute mash.

In 2¾ hours added 24 pounds soda ash.
In 3 hours more added 36 pounds soda ash.
In 2 hours more added 48 pounds soda ash.
In 4½ hours more added 48 pounds soda ash.
In 6½ hours more added 36 pounds soda ash.

He kept the temperature at 30°C. and fermented to quiescence or for about five days. The fermented mash then contained 3.1 per cent glycerin, 6.75 per cent alcohol and 0.86 per cent sugar with an alkalinity of 3.6 grams sodium carbonate per 100 cc.

Connstein and Ludecke (6) discuss glycerin production through Neuberg's third and second fermentation reactions. They tested fermentation under alkaline conditions comparing the effect of various alkalies at varying concentrations and give the following table of results obtained.

	per cent glycerin
Control with no alkali addition gave	3.0
Sodium acetate to 30 per cent of sugar weight gave	9.5
Sodium bicarbonate to 14 per cent sugar weight gave	12.7
Ammonium carbonate to 30 per cent of sugar weight	13.4
Disodium phosphate to 46 per cent of sugar weight	11.0
Disodium phosphate 70 per cent of sugar weight	15.6

They state however that the alkaline conditions increase the danger of infection with lactobacilli and other contaminants, increase the difficulty of determination and lessen yields.

SULPHITE PROCESS

Sodium sulphite lessens the danger of contamination as it acts as a bacterial antiseptic and it gives greater yields, the percentage of glycerin produced increasing with increase in the ratio of sulphite to sugar. All forms of fermentable sugars can be used as can all actively fermenting yeast strains. Equally good results can be obtained with

refined sugar, raw sugar and molasses. They show the relation of sulphite used to glycerin produced.

Sodium sulphite to 40 per cent of sugar gave 21.1 per cent conversion to glycerin.
Sodium sulphite to 67 per cent of sugar gave 24.8 per cent conversion to glycerin.
Sodium sulphite to 80 per cent of sugar gave 28.3 per cent conversion to glycerin.
Sodium sulphite to 100 per cent of sugar gave 30.1 per cent conversion to glycerin.
Sodium sulphite to 120 per cent of sugar gave 33 per cent conversion to glycerin.
Sodium sulphite to 150 per cent of sugar gave 34.6 per cent conversion to glycerin.
Sodium sulphite to 200 per cent of sugar gave 36.7 per cent conversion to glycerin.

Yeasts must be educated to fermentation with the larger concentrations and sulphite must be added by degrees, each time in just sufficient amount to temporarily check fermentation. In our own laboratory we have educated a yeast to ferment in the presence of sodium sulphite to 180 per cent of the sugar used.

Yeast cultures may be used for repeated fermentations especially if a "purifying fermentation" under weakly acid conditions intervenes periodically, thus forming a new active generation. With a yeast so regenerated once they obtain under certain conditions an 18.8 per cent yield of glycerin, after three regenerations the same yeast under the same conditions gave a 22.9 per cent glycerin yield and after 8 regenerations a 21.2 per cent yield.

With 10 liters of a 10 per cent sugar solution, a proportionate amount of the usual nutrient salts, 100 grams of fresh yeast and 400 grams of sodium sulphite as anhydrous sodium sulphite fermenting at 30°C. they obtained complete sugar consumption in forty-eight to sixty hours with very little alteration of pH value.

RECOVERY

At the end of fermentation volatile substances present are distilled off, and then most of the sulphite is removed with calcium chloride and calcium carbonate addition. Excess carbonate is removed with soda, leaving a syrup containing chiefly salt and glycerin. To determine glycerin yield extract a measured volume with alcohol and test

the extract by the Zeissl-Fantose method with isopropyl iodide or by the Benedikt-Cantor acetin process, both described by Lewkowitsch (2), or distill the fermented batch with steam in vacuo into an air-cooled condenser, collecting the watery distillate in a succeeding water cooled condenser.

The volatile distillate will contain acetaldehyde and water. With increasing sulphite addition alcohol and carbon dioxide production will decrease as aldehyde and glycerin increase, as given by the authors last quoted (6) in table 5. The sulphite acts as a salt and also specifically. The increase of any non-toxic salt will increase glycerin to some extent.

By this method the authors report the manufacture of over 1,000,000 kilos of glycerin per month with about 25 per cent conversion of the sugar to glycerin, the balance forming acetaldehyde, alcohol and

TABLE 5

GLYCERIN FERMENTATION

SULPHITE IN TERMS OF SUGAR	ALDEHYDE	ALCOHOL	CARBON DIOXIDE
per cent	*per cent*	*per cent*	*per cent*
25	2.42	39.96	37.6
50	5.80	35.80	35.8
100	10.00	29.40	29.4

carbon dioxide. They state that the method can compete with glycerin production from fats, but this will vary with the cost and availability of raw materials.

In 1924 Connstein and Ludecke (10) were granted a United States patent for glycerin production covering the use of either alkaline salts as ammonium phosphate, carbonate, sodium phosphate, carbonate, bicarbonate, sulphite, or acetate or amine salts. A single compound or a mixture may be used and the amounts varied. Nutrients for the yeast cells may be added if advisable. Magnesium sulphate, iron sulphate, manganese sulphate, or other catalysts may also be added.

MIXED SULPHITES

In 1922 Cooking and Lilly (9) were granted a patent for the production of glycerin by fermentation in the presence of normal sulphites. They employ a mixture of sodium sulphite and bisulphite, the pres-

ence of the latter anticipating the eventual splitting of the normal sulphites resulting in the fixation of the acetaldehyde at an earlier stage and the consequent saving of a portion of the sugar which, with the normal sulphite alone, would be completely changed to alcohol. The alkalinity due to sulphite splitting is reduced to a minimum or may be eliminated. The time is also shortened. With normal sulphite alone,

$$\underset{}{2CO_2} + \underset{}{2H_2O} + \underset{252}{2\,Na_2SO_3} = \underset{}{2NaHSO_3} + \underset{168}{2NaHCO_3}$$

and with the mixed salts in molecular proportions,

$$\underset{}{CO_2} + \underset{}{H_2O} + \underset{230}{NaHSO_3} + \underset{}{Na_2SO_3} = \underset{}{2NaHSO_3} + \underset{84}{NaHCO_3}$$

By varying sulphite and bisulphite additions the relative amounts of glycerin and alcohol produced may be varied.

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CHAPTER 13

Acetone and Ethyl Alcohol Production

INTRODUCTION

While the amylobacters were primarily used to produce acetone with butyl alcohol as an important by-product, they are not the only organisms that will form this substance from carbohydrates. As early as 1906 Breaudat (1) described an acetone fermentation of saccharose by a *Bacillus violarius acetonicus* but the amount produced in his experiments was very small and as far as we can find, this was never used industrially.

As a result of the great demand for acetone during the world war and of the generally renewed interest in the possibilities of solvent production by microörganisms many investigators sought for new possibilities in this field. J. H. Northrop, working for the Council of National Defense discovered a hitherto undescribed organism capable of fermenting carbohydrate material with the production of ethyl alcohol and acetone. This organism he first isolated in 1917 and named *Bacillus acetoethylicum.* In February, 1919, he patented the "Process for the Production of Acetone" (6) from starchy or sugary material with this organism, assigning the patent to The Rockefeller Institute, by whom it was dedicated to public use.

ACETO-ETHYLICUM-MORPHOLOGY

In the patent claim the organism is fully described according to the descriptive chart of the Society of American Bacteriologists, as follows:

I. Morphology

1. Vegetative cells, motile:
   (a) Medium used—agar slant, or 10 per cent corn medium (1 part corn in 10 parts water), temperature 40°C., age twenty-four hours.
   Form—short rods, no chains in agar slant. Occasional short chains in 10 per cent corn medium.
   Size—4–6μ x 0.2–0.3μ.
   Ends—rounded.
   Stain—evenly with Loeffler's methylene blue or gentian violet. Gram stain negative.

(b) Medium used—10 per cent corn medium, temperature 40°C., six to ten days.
Form—short rods—occasional short chains.
Size—4–6μ x 0.2–0.3μ.
Ends—rounded.
Stain—unevenly with Loeffler's methylene blue or gentian violet, with deep stained spot at end or in center. Gram stain negative.

2. Sporangia:
Medium used, glucose agar slant.
Temperature—40°C., after two to three days spores formed.
Form—elliptical. Spores form at end of rods.
Limits of size—1μ.
Size of majority—0.6–0.8μ.
Spores stain easily with Loeffler's methylene blue.

II. Cultural features

1. 2 per cent glucose agar slant medium, age twenty-four hours, temperature 40°C.
Growth, moderate.
Form of growth, spreading.
Elevation of growth, effuse.
Luster, dull.
Optical character, translucent.
Odor, absent.
Medium, clear.
Condensation water, opaque.

2. Potato, twenty-four hours, 40°C.
No visible growth on surface.
Gas bubbles all over medium, crumbles easily, no odor.
After two to three days, 40°C.
Medium sinks to grayish white paste.

2a. Glucose broth. After twenty-four hours, 40°C. (Medium cloudy, no odor.)
Slight gas bubbles.
After two to three days, slimy mass in bottom of test tube.

4. Litmus milk, twenty-four hours, 40°C.
Bottom of tube reduced to white, no gas, odor, acid, or clot.
Thirty-six hours, 40°C. Milk red on top, rest white.
Seventy-two hours, 40°C. Same but coagulated; clot does not digest subsequently.

10. Agar colonies. 2 per cent glucose agar, twenty-four hours, 40°C.
Growth, slowly spreading.
Round, sometimes irregular.
Surface, smooth.
Elevation, effuse.
Edge entire or undulate.
Internal structure, coarsely granular.

15. Sodium chloride in bouillon.
    Inhibiting concentration is 4 to 5 per cent, sodium chloride.
17. Nitrogen source. With sugar as carbohydrate may be obtained from peptone, proteins or ammonium salts.
    With starch. Same but cannot use ammonium salts.
18. Best medium for long continued growth, 2 per cent corn medium with $CaCO_3$.

III. Physical and biochemical features

1. Fermentation tubes:
   See table of sugars fermented, infra.
7. Optimum reaction of media:
   For growth, Sorensen's pH values—8.0–9.0.
   For fermentation, Sorensen's pH values—6.0–8.0.
8. Vitality on culture media:
   At least six months at room temprature.
   At least three months at 40°C.
9. Temperature relation:
   Optimum temperature 40°C. to 43°C.
   Spores may be boiled at least twenty minutes.
10. Resistant to drying.
13. Acids produced, formic.
15. Alcohol, ethyl, propyl and butyl.
    Ketones, acetone.

IV. Pathogenicity

1. Non-pathogenic to mice.

ISOLATION

Northrop, Ashe and Senior (7) describe the isolation of the organism in the following way:

Potato cylinders in test tubes are sterilized in the Arnold for twenty minutes to kill less resistant forms. They are then incubated for several days at 40°C. and examined for crumbling and evidences of gas formation. Those which are gassy and have completely crumbled but have developed no odor are covered with water, distilled and the distillate tested for acetone with para-nitro-phenyl-hydrazine, after making smears for staining and plating in 2 per cent glucose agar. If this organism is present needle-like golden-yellow crystals will form in the distillate on treatment with the above reagent. The stained smears will show long slender rods with large oval spores and the glucose agar plates will show small transluscent colonies. The organisms are fished from such colonies and replated twice again in 2 per cent glucose agar. Colonies from the third series of plates are transferred to glucose agar slants and other media for systematic study and identification according to above description.

The organism is a facultative anaerobe and resembles *Bacillus macerans* except that it ferments galactose and levulose anaerobically with ammonia salts as the only source of nitrogen.

TESTING FERMENTATION ACTIVITY

To further test the ferment activity of the organisms isolated pure cultures are inoculated into sterile flasks containing 10 grams of peptone, 100 grams of potato starch and 20 grams of calcium carbonate in 2 liters of water. After inoculation the cotton plug is covered with tinfoil to prevent evaporation. After four to eight days incubation at 40°C. the product may be distilled and tested with para-nitrophenyl-hydrazine or with iodoform, or precipitated with mercuric sulphate.

The paper referred to above also gives tests for identifying alcohol and formic acid. For details of the tests see *Journal of the American Chemical Society* (5), (10).

Contaminating organisms destroy the acetone as formed. The volatile products of the fermentation inhibit the activity of the organisms, so parent cultures should not be sealed. Continuous fermentation in closed vessels is only possible for short periods of time. In fermenting cultures considerable slime forms and settles out, entraining the organisms and slowing their activity.

ADDITION OF INERT MATERIAL

To minimize this effect it is advisable to have mashes containing considerable inert solid material, as twigs, corncobs, coke or broken marble on which the slime will accumulate, allowing the liquid containing the bacilli to drain off. If this is done fermentation will be completed in fifty to sixty hours, where otherwise it would require a week or more.

SEMI-PLANT SCALE TESTS

Northrop, Ashe and Morgan (8) describe the original experimental work preceding fermentation on a plant scale. Ten-gallon tin lined copper vessels were used. A beet molasses was prepared by acid hydrolysis and neutralization. This contained 1.05 grams of sugar as dextrose per cubic centimeter and was diluted to 15 volumes with water, giving a concentration for fermentation of 70 mgm. sugar per cubic centimeter. It was sterilized for four hours at 15 pounds pres-

sure. One gallon of this mash was inoculated with the growth from five glucose agar slants and incubated for twenty-four hours. This was then used as inoculum for 7.5 gallons of sterile mash containing broken marble. In fifty to sixty hours the pH value fell from 8 to 6, acetone was present equivalent to 8 to 8.5 per cent of the original sugar and ethyl alcohol representing 20 to 21 per cent of the same. The fermented mash was withdrawn and it was possible to ferment four 5-gallon refills without inoculation. The second series of tests used 160 to 175 gallon amounts of more dilute mash adjusted to a pH value between 8.5 and 9.5 before inoculation, and with brush as the inert material. For every 5 gallons of molasses they added 120 gallons of water and 5 pounds of air slaked lime.

END PRODUCTS

In addition to ethyl alcohol and acetone formic acid and traces of propyl and butyl alcohol were identified. Aertzberger, Peterson and Fred (11) working with the same organism state that from 90 to 95 per cent of the yield from sugars is made up of acetic, formic and lactic acids, ethyl alcohol, acetone and carbon dioxide and that 75 to 80 per cent of the sugar is feremented in from ten to fifteen days. Volatile acids formed consist of about 45 per cent formic and 55 per cent acetic acid. Small amounts only of lactic acid are produced and traces only of the higher alcohols. Ethyl alcohol production represents from 8 to 25 per cent of the sugar fermented. The optimum pH value for solvent production they found to be from 5.8 to 6. An alkaline pH value of 8 or over favors the formation of large amounts of volatile acids with low solvent yields.

The acetone production is only apparent after several days fermentation.

CARBOHYDRATES ACTED ON

They state that the organism will ferment glucose, sucrose, potato starch, and xylose in a peptone-phosphate medium. For experimental work they used the following medium:

	grams
Peptone	5.00
Di-potassium hydrogen phosphate	1.00
Carbohydrate	20.00
Water	974.00 cc.

The patent claim (6) gives the yields from various carbohydrates as shown in table 6.

Although the patent claims provide for the addition of nitrogenous nutrient material and a buffer substance, yet they state that with

TABLE 6

TABLE OF SUGARS FERMENTED

SUBSTANCE	ACETONE	ALCOHOL
	per cent	*per cent*
Galactose*	4 to 5	22 to 24
Maltose	6 to 7	23 to 24
Mannose	6 to 7	22 to 23
Raffinose	8 to 10	22 to 23
d-Arabinose	6 to 7	12 to 16
Ca-lactate	—	—
Starch	8 to 10	20 to 24
Beet molasses	8 to 10†	16 to 24†
Potatoes, white or sweet	2 to 4‡	4 to 9‡
Dextrin	6 to 7	14 to 16
Dextrose	9 to 10	22 to 23
Levulose*	8 to 10	24 to 25
Xylose	4 to 5	18 to 20
Glycerin	—	40 to 43
Sucrose	8 to 9	24 to 26
Corn	10 to 13	20 to 26
Corn cob	1 to 5	8 to 10
Horse chesnuts	7 to 8	14 to 17

* Ferments also under following conditions, with same percentage yield of acetone and alcohol:

	grams
Potassium phosphate	1.00
Ammonium phosphate	1.00
Sodium chloride	trace
Calcium carbonate	2.00 to 5.00
Galactose or levulose	10.00
Water	1000 cc.

Fermented at 40°C., in a Gruber's anaerobic tube. Fermentation complete in six to seven days.

Dextrose, levulose, galactose, cane sugar (sucrose), maltose, lactose, starch, *d*-arabinose also ferment with the above solution in a flask of such size that the liquid forms a shallow layer on the bottom.

† Volume per cent.

‡ Per cent of fresh weight.

corn and molasses, the two materials used in the examples of plant processes given, neither nitrogen or buffer additions are necessary if inert materials, as marble chips, are used.

REFERMENTATIONS

In the patent a fill and draw method was used permitting of several refermentations from each inoculation. The first fermentations were complete in four days, but succeeding fermentation were complete in forty hours.

XYLOSE FERMENTATION; CORN COBS

In 1921 Peterson, Fred and Verhulst (12) reported experimental work on the fermentation of a xylose syrup, from hydrolyzed corn cobs as a cheap source of carbohydrate, with this organism. Sulphuric acid to 8 per cent of the weight of cobs with 40 volumes of water was used with a four-hour autoclaving at 20 pounds pressure. A syrup was obtained which, on neutralization with calcium hydroxide, pressing and washing the insoluble residue, contained the equivalent of 26.7 per cent of the cob weight as sugar. This was diluted to 3 per cent sugar calculated as dextrose. The reaction was adjusted to a pH value of 7.6 to 8.4 and the materials were then sterilized in flasks containing coarse cinders as inert material and an excess of calcium carbonate. The flasks were inoculated and covered with tinfoil to prevent solvent loss. Several fillings were fermented in these flasks without reinoculation. One hundred pounds of cobs yielded on an average of 25 pounds of sugar of which 22.5 pounds was fermented with a yield of 2.7 pounds of acetone, 6.8 pounds of alcohol and 3.4 pounds of volatile acids.

HULLS

In 1923 Fred, Peterson and Anderson (13) reported the fermentation of hydrolyzed oat and peanut hulls by the same method. The hulls were autoclaved for two hours at 15 pounds pressure with 2 per cent sulphuric acid present. The mass was then neutralized with milk of lime, pressed and washed. The xylose syrup from the hulls contained 26.5 per cent of sugar, as glucose, and that from peanut hulls 7.6 per cent. The syrups were diluted to about 3 per cent sugar content as glucose and fermented with *Bacillus acetoethylicum* with inert matter and calcium carbonate and the addition of a small amount of

peptone and sodium phosphate. Maximum gas evolution by the third or fourth day made a foam layer over the surface 2 or 3 inches thick. Gassing ceased in ten days with almost complete fermentation of the reducing sugars. Oat hull syrup with an initial glucose value of 3.22 per cent after seven days fermentation was reduced to 0.44 per cent sugar with the formation of 0.47 per cent of acetone, 0.876 per cent of ethyl alcohol and 0.169 per cent of volatile acids. Peanut hulls syrup with a 2.43 per cent glucose value after eight days was reduced to 0.462 per cent sugar with 0.314 per cent acetone, 0.546 per cent ethyl alcohol and 0.436 per cent volatile acid. There was a calculated yield from 100 pounds of oat hulls of 3.9 pounds of acetone, 7.2 pounds of alcohol and 1.4 pounds of volatile acids.

The same crude xylose syrups diluted with yeast water (13) to 2 per cent sugar as glucose and inoculated with *Lactobacillus pentoaceticus* underwent a slow fermentation incomplete in thirty days with a low solvent yield.

THEORY OF THE FERMENTATION

Bakonyi (14) discusses the theory of the fermentation by *Bacillus acetoethylicum.* He finds that with high sugar concentrations (4 to 5 per cent) more acetone is produced and with low sugar content more alcohol. He proved that acetaldehyde may be an intermediate product by showing that the organism can readily ferment acetaldehyde and that acetaldehyde entrainment occurs with the addition of $NaHSO_3$ to the cultures. The fermentation is analogous with ordinary alcoholic fermentation up to the stage of acetaldehyde production. Then the three carbon acetone is formed from the two carbon acetaldehyde by aldol condensation.

$$2\ CH_3CHO = CH_3CHOH \cdot CH_2CHO$$

Acetaldehyde — Acetaldol

Acetaldol probably takes up one molecule of water to form acetic acid and alcohol.

$$CH_3 \cdot CHOH \cdot CH_2CHO = CH_3CHOH \cdot CH_2\overset{H}{\underset{OH}{C}}OH =$$

Acetaldol

$$CH_3CH_2OH + CH_3COOH$$

Alcohol — Acetic acid

As calcium acetate is completely fermentable to acetone acetic acid may be an intermediate. The acetic acid united with calcium from the calcium carbonate present to form calcium acetate which is changed to acetone.

$$2\ CH_3COOH + CaCO_3 = Ca(CH_3COO)_2 + CO_2 + H_2O.$$

$$Ca(CH_3COO)_2 = CH_3COCH_3 + CaCO_3.$$

This would seem to be a useful fermentation with commercial possibilities provided that there was available an abundant supply of cheap carbohydrate material.

ACETONE FROM MOLD ACTION

In 1915 Fernbach and Strange were granted an English patent (4) for the production of acetone, acetates and pyruvates by fermentation of a starchy or sugary mash with a mold, *Amylomyces rouxii*, in the presence of an alkaline earth metal carbonate, the fermenting mash to be aerated with sterilized air or oxygen. At the end of the fermentation period there will be obtained a mixture of salts, mainly pyruvates and acetates which are removable by filtration. The filtrate may then be concentrated or precipitated with alcohol. The resulting mixture of salts may be oxidized entirely to acetates which may be used for the production of acetone or acetone may be produced by destructive distillation of the unoxidized salt mixture.

The above method seems to be a modification of the amylomyces process for alcohol production (2) patented in France by Fernbach in which the same organism is used in conjunction with a yeast to produce alcohol from starches. The yeast in the latter process is added after the mold action is established and by its abundant production of carbon dioxide gas prevents the further use of the alcohol by the fungi. In the former process the alcohol appears to be an intermediate in the acid production.

The fungi employed is isolated from Chinese yeast (3) and is present with the yeast because of its diastatic action. This yeast is used in China, Cochin China and neighboring countries for the preparation of rice spirit and is on the market in the form of flat mealy balls about the size of half a crown.

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Section III

THE COMPLEX NITROGENOUS MATERIALS

CHAPTER 14

Proteins, Glue and Gelatin

PROTEINS

Chemically proteins may be regarded as combinations of amino acids or their derivatives, while their physical properties are due largely to their colloidal nature. Protein substances are essential constituents of all living cells, plant and animal.

PROTEOLYTIC BACTERIA

Proteins are decomposed by aerobic, anaerobic and facultative bacteria and some molds. Industrial application of this specific action is found in the degumming of silk, manufacture of cornstarch and cocoanut oil and the removal of hairs from hides. The clostridia are rapid liquefyers under anaerobic conditions, the proteus group are facultative organisms and the bacilli are generally aerobes. The green fluorescent bacteria, Pseudomonas, are also active liquefyers. The action is facilitated by aeration.

Genera containing strongly proteolytic bacteria are:

Clostridia, anaerobes or microaerophiles, rods enlarged at sporulation, Gram positive, generally motile, will attack native protein in absence of carbohydrates. Types: *Clostridium putrificum.*

Bacillus, aerobic and facultative rods, spore formers, Gram positive, generally motile.
Type: *Bacillus subtilus.*

Proteus, pleomorphic rods, Gram negative, generally motile, facultative, non-spore formers.
Type: *Proteus vulgaris.*

Pseudomonas, small rods, non-spore formers, Gram negative, generally motile, facultative.
Type: *Pseudomonas fluorescens.*

These organisms are readily isolated because of their wide distribution in nature, namely, soil, water, dust, feces and decaying meats. They grow profusely on the ordinary culture media. Litmus milk is first coagulated and then digested. Anaerobic spore formers may be isolated by placing garden soil in tubes of litmus milk

overlaid with mineral oil and heating at 85°C. for twenty minutes with subsequent incubation at about 35°C. for twenty-four hours.

PROTEOLYTIC PROCESSES

Trotman and Sutton (1) use *Bacillus subtilus*, for the removal of protein from cotton fibers and also refer to the use of *Bacillus mesentericus*. Waksman (2) recommends *Bacillus florescens liquifaciens*, *Pseudomonas fluorescens*, and *Bacillus mycoides* as type organisms in a process for removing silk gum or sericin from silk fibers. The process was patented in 1922.

Sericin or silk gum has the following composition:

	per cent
Carbon	48.8
Hydrogen	6.2
Oxygen	26.0
Nitrogen	19.0

The sericin when treated with a mixture of oil and soap at 90° to 95°C. for half an hour and subsequently washed with warm water or steam is subject to the action of proteolytic bacteria. The raw silk fiber after such treatment is transferred to a suitable vat containing a nutrient peptone salt medium, which is inoculated with proteolytic bacteria. An incubation period of twenty-four hours at about 35°C. is sufficient for removing the sericin. The pure silk fiber is not attacked. A later patent by Wallerstein, in 1927 (3), uses papain for the proteolytic fermentation at 50°C.

The proteolytic action of bacteria on an extract of pressed cocoanut meats after they are clarified and separated into an aqueous portion and a creamy emulsion of albumin and oil was patented by Alexander (4). The emulsion portion is pasteurized at 63°C., cooled to 36°C. and run into ripening vats where inoculation with proteolytic bacteria capable of digesting the albumin occurs. This is followed by incubation at 35°C. for about ten hours to complete the digestion. The addition of weak alkali facilitates separation of the dry cocoanut oil. According to the patent claim suitable organisms may be had by allowing the emulsion to stand at 35°C. over night.

Dernby and Blanc (5) found that the filtrates from *Clostridium sporogenes* and *Clostridium histolyticum* would liquefy gelatin and disintegrate peptone in a pH range from 4 to 8. They conclude that tryptase is present.

Cornstarch may be rid of its residual protein by the action of proteolytic bacteria according to the patent of Lenders and Allen (6). The process consists of mixing the starch with hot water, 100°C., to a density of about 21°Baumé. The suspension is inoculated with proteolytic bacteria and the contents of the vat stirred for about two hours. The starch is allowed to settle out over a period of twenty hours. The supernatant liquid is decanted and fresh water added, the process being repeated several times. The starch liquor should have about 2,000,000 bacteria per cubic centimeter for best results. *Bacillus putrificus* is stated as the type organism but according to the *Manual* this organism belongs to the genus Clostridium, an anaerobic bacterium. Since the patent claims call for aeration it is quite likely that other bacteria, namely, aerobes are responsible for the digestion.

In the manufacture of grape sugar the starch may be treated prior to conversion by proteolytic bacteria as stated above. Allen claims (7) an improved method for the manufacture of grape sugar by using the bacterial digestion as the means of purifying the starch.

A method for deliming wool, hair or bristles by the action of aerobic proteolytic bacteria was patented by Lucas in 1917 (8). Suitable nutrients are added to the lime liquors and the fermentation temperature is maintained at approximately 30°C. The patent states type organism as *Bacillus boocopricus* and *Bacillus subtilus*.

Effront and Boidin claim (9) a process for the manufacture of pressed yeast in which the amylaceous material is subject to the action of proteolytic bacteria until the complex nitrogenous substances are rendered assimilable.

GLUE AND GELATIN

Glue is a decomposition product made by heating animal tissues up to about 100°C. but not boiling the mass. Gelatin is made according to the same process but a better grade of tissue is more carefully processed. Hide glue is prepared from tissue generally referred to as green stock. This stock consists of hide trimmings, scrapings, untanned waste, slaughter house waste, ear laps, heads, sinews, feet, tails and scrap flesh. It is limed and allowed to stand in large piles for several months. During this period if it is not carefully protected a large amount of deterioration may take place resulting in low glue yields that may be attributed to later stages in the process of

manufacture. Piles of old and new stock should not be mixed and it is preferable to run through batches of old limed stock separately. The bones of beef and calves are used also in the preparation of glue. The need of adequate treatment of the raw stock to prevent deterioration and all sanitary precaution during the process of manufacture can not be over emphasized.

Dry glue may contain about 15 per cent moisture. Zinc sulphate is a common preservative although alum, borax, salicylic acid and formalin are also used. It is preferable to use a preservative that is odorless. If starch is added to glue to make a glue paste the mixture may ferment with marked foaming.

Crude carbolic acid is the most useful disinfecting solution to use around the plant. It will kill spore-forming rods in a 10 per cent solution and non-spore formers in about a 3 per cent solution. Its phenol coefficient is about 2.75.

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CHAPTER 15

Leather, Hides, Tanning, Salt Stains, Anthrax, and Artificial Leather

THE LEATHER INDUSTRY

Bacteria and molds may play very important rôles in various processes connected with the tanning of skins and hides. On the one hand bacteria may take a very essential part in the preparation of skins for tanning and molds may be essential in the preparation of some vegetable tanning liquors. On the other hand both bacteria and molds may be responsible for serious and irreparable damage to skins before they reach the tan liquors, and the bacteria may be a menace to the health and life of the tanner.

BENEFICIAL ACTIONS

In another chapter we have discussed the function of certain molds in the ripening of tanning solutions from gall nuts. Skins and hides after removal from the animal carcasses must be salted to preserve them until ready for tanning. On reaching the tannery the salted skins must be soaked in fresh water to remove the salt, milled in revolving drums to soften, fleshed to remove excess fat and other organic material, and then dehaired by soaking in strong lime suspensions with or without the addition of other substances. The lime renders the skin alkaline, softens it and loosens the hair so it may be scraped off. Wood and Law (2) regard the growth of bacteria as an essential part of the dehairing process due to the proteolytic action of bacterial enzymes and Waksman and Davison (13) say that both proteases and lipases are active in unhairing. In the older sweating process, where the skins were hung in a hot humid room until the hair loosened, the action was entirely due to bacteria. The lime or the bacteria or both combined dissolve the superficial epidermis, loosen the hair and separate the fibers of the corium.

After dehairing, the skins are washed and then bated or pured. Formerly this was done by soaking in suspensions of dog or bird dung, the action being due to the various intestinal and bacterial enzymes

present. These act to dissolve out the elastin fibers, leaving the collagen to absorb the tan liquors and be changed to leather. The excretal products are still used in some tanneries for bating but they are being largely replaced by solutions of pancreatin and bacteria and bacterial enzymes or, in some instances of protease prepared from molds such as *Aspergillus flavus-oryzae* or *Aspergillus wenti* (9), the preparation of which is described in another chapter. According to Wilson and Merrill (12) the limed skin with its manifold and various protein degradation products may be acted upon by enzyme bate liquors, with their countless bacteria in many different ways. They list the purposes of bating as:

1. Rendering the skin flaccid.
2. Adjusting the hydrogen ion concentration.
3. Removing lime.
4. Removing elastin fibers.
5. Partial digestion of collagen.
6. Removal of dissolved keratin.
7. Removal of "scud" or unorganized material derived from epithelial tissues.

Commercial enzyme preparations are usually a mixture of several enzymes.

After bating skins may be or may not be drenched by soaking in a bran emulsion. The carbohydrates in the bran ferment with the production of various organic acids and carbon dioxide gas. If acid fermentation does not occur an alkaline proteolytic decomposition may ruin the skins if they are not removed promptly (10).

HARMFUL ACTIONS

While the necessary actions of dehairing and bating may be due to bacterial proteolytic enzymes, yet if similar proteolytic enzymes act irregularly and over longer periods on local areas of untanned skins they may seriously injure them or completely destroy their value. This occurs when skins are unevenly and insufficiently salted on removal from carcasses or when salting is delayed.

A trade article (14) calls attention to serious damage due to the development of pink, red or black spots on hides due to the action of proteolytic organisms. This is termed "heating" in the trade and is due to bacterial action. More active putrefaction may also occur with the development of blue or green slimy areas. These dis-

colored areas show as weak spots or holes in the tanned skins. We have ourselves isolated spore-forming rods with very active proteolytic and reducing enzymes from such damaged skins where tanned hides from the same source were developing decidedly weak spots.

This spottiness will not occur if hides are properly salted as soon as removed from carcasses. A good practice is to put the fresh hides immediately into brine to cool and after some hours soaking, to salt thoroughly and evenly with dry salt. We find proteolytic action may be arrested on receipt of partially damaged hides by soaking twenty-four hours, or milling a shorter time, in a disinfectant such as a 1 per cent pine oil emulsion and then resalting, but this is expensive and will not remedy damage already done.

TABLE 7

ANTHRAX CASES AND DEATHS IN THE UNITED STATES, BY SOURCES OF INFECTION, JANUARY 1, 1927 TO JULY 1, 1929

	CASES	DEATHS
Tannery and leather infections	47	3
Wool infections	43	4
Hair and brush infections	11	0
Handling livestock	21	7
Laboratory infections	1	1
Source not reported	59	9
Totals	182	24

Thom and Church (11) state that *Aspergillus niger* may be a serious destructive agency in the tanning industry, especially as it is so tolerant of tan liquors. It may produce discoloration showing in finished leather due to structural change causing a changed reaction to finishing processes. He also found *Penicillium rugulosum* (16) and other species of Penicillia on damaged hides.

ANTHRAX IN INDUSTRY

The senior author has a number of times called attention to the anthrax hazard in industry. We quote from his 1929 report to the Industrial Hygiene Section of the American Public Health Association (17) (see table 7).

Anthrax is a continuing menace in the tanning industry and an

increasing one in wool handling. The latter fact is probably due to the diversion of low grade wool to this country due to English disinfection requirements. At present there is comparatively low fatality from tannery anthrax, due to constant watchfulness and prompt intelligent treatment, and an alarmingly high fatality in anthrax due to animal contact, possibly due to the rapid spread of many actively growing vegetative bacilli introduced into wounds as compared with the longer incubation necessary for the development of the comparatively few spores derived from the handling of hides, skins, or hair.

Skins and hides may be disinfected for anthrax by:

1. The Schattenfroh method (1), consisting in a twenty-four-hour soak in a 10 per cent sodium chloride solution containing 3 per cent hydrochloric acid.

2. The Lehman method (7), consisting in a twenty-four-hour soak in a 10 per cent sodium chloride solution containing 5 per cent sodium carbonate.

3. The Seymour-Jones method (6), consisting in a twenty-four-hour soak in 1:1000 bichloride of mercury containing 1 per cent formic acid.

The American tanners claim that the Schattenfroh method makes leather too harsh, although it is used routinely abroad. The Lehman method is not used in this country as far as we know, but we believe it to give good results; however, skins so soaked must be tanned at once or they will deteriorate so it can only be used in the tannery and cannot be used at a shipping port. The Seymour-Jones method seems to be effective provided that the skins are held several days after soaking, as the rate of spore death is slow.

We have successfully killed anthrax spores in heavily infected skins by iodine vapor, iodine-carbon tetrachloride solution and iodine, potassium iodide watery solution with subsequent recovery of unused iodine with sodium thiosulphate, but the present cost of iodine makes the method prohibitive. The lime soak, 15 pounds of lime or calcium hydroxide per 100 gallons of water, or the 1:1000 bichloride of mercury soak recommended in the Bureau of Animal Industry regulations (3) we have found not dependable.

Anthrax infection menace in wool or hair may be removed by autoclaving, by a twenty-four-hour exposure, not less, to 200°F. dry heat (8) or by the English method of washing in hot alkaline suds followed by hot formalin solution and several days subsequent storage. All

wool and hair imported into England from countries where anthrax is prevalent must be submitted to this process at a government disinfecting station in Liverpool (4).

Although anthrax in animals is already indigenous over large areas in this country as yet practically all of our industrial anthrax is traceable to imported infected raw materials. So we are hoping to see the day when all such imported material will be compelled to pass through one or one of several government disinfecting stations or better still follow the English suggestion of passing through internationally controlled disinfecting stations at shipping ports in the countries of origin. We realize that to many this idea seems utopian, yet it would result in anthrax being practically excluded from the manufacturing industry.

SALT STAINS

Light yellow, rust brown, greenish blue, and violet stains that appear on hides and skins are termed "salt stains." Usually these stains are not noted until at or after unhairing. They are intensified and often darkened by sulphide-lime liquors and vegetable tan liquors and sometimes appear on the grain as crust-like defects of irregular shape and distribution. Due to the difficulty of detection on the green salted hides before the hair is removed and the fact that much of the leather on which they are found must be worked into cheaper grades, these salt stains are the cause of much concern to the tanner and of a real economic loss to the producers of hides and skin and to the makers and users of leather.

The cause of such stains has long been a moot question. An interesting description of them is given by Abt (19) as follows:

> In the skin with the hair on, there appear on the flesh side small rust colored spots, with the consistency of a damp crust. After unhairing, they show on the grain, where they form raised designs of festoon shapes, slightly yellow in color and during tannage the color becomes gray, black or dark brown.

Abt attributed these stains to the presence of "schlott" or calcium sulphate in the salt and maintained that most of the stains which he had found were of this origin. He conceded, however, that bacteria might play an important part in the formation of stains of other types.

Becker (20) isolated cultures from orange, yellow and red stains and found them capable of producing corresponding stains in pure culture on various media.

Jourve (21) gives as the causes of such stains: (1) Liquefying bacteria; (2) iron; (3) acid swelling provoked by magnesium chloride in the salt.

Paessler (22), (23), (24) repeatedly points out that salt stains are not due to a single cause. He considers them generally as the result of two different causes, namely, impurities in the salt and bacteria.

Rappin, Grosserone and Sonbranne (25) consider bacterial decomposition as a contributing factor in the formation of stains. Turnbull (26) attributes their formation to six sources, among which he lists chromogenic microörganisms and their action in the presence of calcium and magnesium sulphates. Eitner (27) includes, among other causes, the putrefactive action of salt-tolerant bacteria as one of the agents contributing to the formation of salt stains. Moeller (28) pointed out that the causes of salt stains could be exceedingly numerous. He assumes that the basic cause is fermentative splitting and hydrolytic decomposition and describes one type of salt strain that may be produced by tyrosine.

Vourland (29) points out that salt stains are usually found on skins from the interior of the pack and are formed without putrefaction or hair slip. He states that the stains are caused by the precipitation of iron from blood by bacterial action.

Romana and Baldracco (30), Schmidt (31), Lloyd (32), Yocum (33), (34), Becker (20), Paessler (23), and others have contributed experiments relative to the elimination of stains. By thoroughly washing and cleaning hides prior to salting and by the use of pure salt with various denaturants they have found that the amount of staining could be considerably reduced.

Recently Bergman and Stattier (35) have associated *Micrococcus pyogenes albus*, *M. citreus* and *M. aureus*, *Bacillus mesentericus*, *fuscus* and *vulgatus*, *Sarcina alba*, *Corynebacterium* and two species of *Actinomyces* with the formation of salt stains.

Summing up the work of various investigators we find bacterial fermentation is universally conceded as being a contributing cause to the formation of salt stains. The actual nature however of such bacterial action is still questionable. Although many theories have been advanced it would appear that much more work is necessary in studying the conditions under which stains occur and the growth of microörganisms under such conditions before any of them can be accepted with a degree of finality.

LEATHER SUBSTITUTES

We find a curious United States patent (18) dealing with the formation of a leather substitute made by tanning a mold or bacterial pellicle growth.

The organism, either *Bacterium xylinium*, *Bacterium xylinoides* or *Mucor boidin* is grown on a suitable culture medium in a large flat shallow vessel. The medium suggested is beer wort containing about a half of 1 per cent alcohol. The size and shape of the vessel determines the size and shape of the finished product. The thickness of the leather to be formed is determined by the length of time the culture is allowed to grow. A culture may attain a thickness up to 30 cm. which will make a finished skin of 0.1 to 0.2 cm. thickness.

To increase firmness and density it is suggested that textile fibers or powdered cork may be floated on the culture medium to serve as reinforcement in concrete or the biological pellicles may be pressed into fabric before tanning. Again a filler of resin soap may be worked into the tanned skin. Fats or oils may be used to soften the skins.

After growth the liquid is drained off, the film treated with a weak lye or 5 per cent sodium sulphite solution and then tanned as would be leather by vegetable tan liquor, alum or chrome methods. We do not know if this process is in actual use commercially.

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Section IV

THE CARBOHYDRATE MATERIALS

CHAPTER 16

Cellulose, Lignocellulose, Wood, and Humus

CELLULOSIC MATERIALS

Several forms of cellulose are recognized by the chemist. The cotton fiber is practically pure cellulose. On hydrolysis normal cellulose yields glucose whereas the hemicelluloses yield hexose monosaccharoses, mannose and galactose and also pentose monosaccharoses. The compound celluloses such as lignocellulose consist of matured cellulose which has been changed by physical combination with gums and resins. Before the compound cellulose undergoes microbial fermentation the cellulose part must be separated from the non-cellulose portions.

A possible triglucose configuration for cellulose, according to Irvine and Hirst (1), is represented in the following structural formula but the true structure is not known.

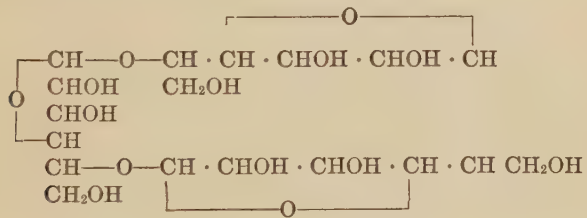

Table 8 shows the approximate distribution of the compound celluloses in nature.

BACTERIA DECOMPOSING CELLULOSE

Cellulose is attacked by bacteria under aerobic and anaerobic conditions. The end products are determined by the microflora and the conditions under which the fermentation proceeds. The organisms used for such fermentations belong to one of six recognized genera:

Cellulomonas: Small rods, rounded ends, non-spore formers, motility varies, aerobic and facultative anaerobes, Gram negative. Grow well on ordinary culture media, found in soil and decomposing vegetable materials, no gas formed (9).

Clostridia: Large rods, spore formers, plectridium and clostridium forms, anaerobes or microaerophiles, generally Gram positive, acid alcohol and gas formed on carbohydrates, found in feces, stall manure and soil.

Actinomyces: Organisms show a branched mycelium, non-motile, generally aerobic or microaerophilic, generally Gram positive, found in soil.

Cytophaga: Rods, long and flexuous, pointed ends, metachromic granules, large spheroid forms observed, aerobe and facultative, motile, Gram negative, cellulose only carbohydrate suitable for use, found in soil with decomposing vegetable tissues (18).

TABLE 8

DISTRIBUTION OF COMPOUND CELLULOSES IN NATURE

	ASH	MOISTURE	CELLULOSE	WAX OIL	RESIN	WATER SOLUTION	PROTEIN	PECTOCELLULOSES MATTER	LIGNIN
Pectocelluloses									
Cotton	0.8	7.0	90.0	0.4	—	0.5	0.7	0.6	—
Ramie	5.4	10.0	66.0	0.6	—	8.0	—	10.0	—
Flax	1.0	9.0	80.0	2.0	—	5.0	—	3.0	—
Hemp	0.8	9.0	78.0	0.6	—	3.0	—	8.6	—
Manila	1.0	12.0	65.0	0.6	—	1.0	—	20.6	—
China grass	2.0	9.0	78.0	0.2	—	6.8	—	6.0	—
Lignocelluloses									
Jute	1.2	11.0	66.0	0.6	—	2.2	—	—	19.0
Wheat straw	4.0	12.0	48.0	1.0	—	6.0	3.0	—	26.0
Esparto	3.5	9.0	48.0	2.5	—	9.0	—	—	28.0
Bamboo	5.0	9.0	53.0	0.8	—	11.0	—	—	26.0
Approximately woods	0.5	11.0	50.0	1.0	2.0	3.0	1.0	—	32.0

Cellvibrio: Long rods, slightly curved, rounded ends, deeply staining granules, polar flagellum, Gram negative, oxidize cellulose, found in soil and vegetable fibers.

Cellfalcicula: Short rods, pointed ends, metachromic granules, polar flagellum, oxidizes cellulose, Gram negative, found in soil.

METHODS OF FERMENTATION

The direct fermentation of cellulose under aerobic conditions in a neutral medium was patented by Langwell in 1923 (2). A large vat heated from 25° to 50°C. by means of a water jacket is fitted with an

aeration apparatus and a stirrer. Sulphite pulp, precipitated chalk, glucose, ammonium chloride, sodium chloride, potassium phosphate and water are placed in the vat. The suspension is inoculated with stable manure, stirred and allowed to stand for fermentation to proceed. The source of the cellulose fermenters may be fermenting vegetable matter, stable manure, pond scum, sewage or garden soil.

For a fermentation of this type it is necessary to select the groups of cellulose fermenters from several different sources. Rich garden soil and buried garbage are possibly the best sources for these aerobic cellulose fermenters. Omeliansky's medium (3) containing inorganic nitrogen is usually a better stimulant of bacterial activity than is organic nitrogen, as peptone. We have found the following medium satisfactory for isolation of these organisms:

	grams
Ammonium sulphate	1.0
Dipotassium phosphate	1.0
Magnesium sulphate	0.5
Calcium carbonate	2.0
Sodium chloride	trace
Distilled water	1000 cc.
pH value about	7.0

Bacteriological test tubes are filled one-third full with the above medium and a small piece of filter paper, about 1 by 6 mm., protrudes above the surface of the liquid. A small amount of soil or stable manure is placed in each of a series of prepared tubes. When the filter paper shows evidence of disintegration a portion is transferred to a second and a third tube until the time required to dissolve the cellulose is materially reduced and upon microscopical examination a uniform flora is observed.

Another satisfactory method is to use sterile 50 cc. flasks containing the medium and a piece of filter paper crumpled so that a portion protrudes above the surface. As the fermentation proceeds the culture become progressively pure and the time consumed is materially reduced. The fermentation temperature is best at about 30°C. although higher temperatures are compatible with organisms isolated from other sources, as fecal material. The use of a normal bacterial flora from vegetable tissue usually results in a prolonged fermentation while material transferred from a previous fermentation results in increased activity with a better yield of end products.

The isolation of thermophilic cellulose fermenting bacteria can be carried out according to the above procedure using the following medium of Viljoen, Fred and Peterson (4):

	grams
Peptone	5.0
Sodium ammonium phosphate	2.0
Mono potassium phosphate	1.0
Magnesium sulphate	0.3
Calcium chloride	0.1
Ferric chloride	trace
Calcium carbonate	excess
Tap water	1000 cc.
pH value about	7.4

Small strips of filter paper are added to this medium. We find the use of liquid medium more satisfactory than the cellulose agar plate method of isolating cellulose fermenters.

Cellulose agar plates are prepared as follows: To 1 liter of diluted ammonium hydroxide solution, containing 10 parts of ammonium hydroxide, specific gravity 0.9000, to 3 parts of water, add a slight excess of copper carbonate. The mixture is shaken vigorously and allowed to stand overnight. After standing, the supernatant solution of cuprammonium is poured off, and in this is dissolved 15 grams of unwashed sheet filter paper. This solution is diluted to 10 liters, and the cellulose precipitated by slowly acidifying the solution with dilute hydrochloric acid (20 per cent). The liquid is now diluted to 20 liters, left for the cellulose to settle, and then decanted from the precipitated cellulose. This is washed with several changes of water, containing hydrochloric acid, until the washings are free from copper, and then with distilled water until free from hydrochloric acid. The cellulose precipitate is again allowed to settle for several days and is finally made up to a 1 per cent suspension. To 500 cc. of this suspension are added 10 grams of agar-agar and 500 cc. of the Omelianski solution already described.

In a later patent Langwell (5) covers a process of cellulose fermentation within a pH value range of 5 to 9, measured in the bulk of the mash. A value within this range is obtained by addition of water soluble phosphate and a compound of an alkali metal, including ammonia, and an alkali earth salt including magnesium. A thermophilic fermentation is used. Among the end products are acetic acid,

butyric acid, alcohol, carbon dioxide, hydrogen and methane. Examples of such fermentations are taken from the patent. The composition of the mash is as follows:

	pounds
Weight of cobs (calculated as air dry)	28.0
Whitening	10.0
Crystalline sodium phosphate	2.2
Commercially pure potassium chloride	1.0
Commercially pure ammonium chloride	0.5
Sodium bicarbonate	0.5

Water to bring the total volume to 180 liters

This mash contains about 7 per cent by weight of cellulosic matter. The above mixture is stirred, heated to about 60°C. in a fermentation vessel which is preferably aluminium lined and jacketed and maintained at that temperature without further agitation, the vessel being closed. The hydrogen ion concentration is maintained within the desired limits by adding sodium bicarbonate once a day in quantities representing about 1 pound of bicarbonate of soda for every 10 cubic feet of gas evolved, measured at normal temperature and pressure. The total weight of bicarbonate usually required for a charge of the above amount is approximately 5 pounds. After a fermentation of eight days a mixture representing the following is obtained:

	grams per liter
Acetic acid	11.7
Butyric acid	0.3
Alcohol	2.2

Gases about 70 cubic feet.

The gases referred to comprise about 80 per cent of carbon dioxide and the remaining 20 per cent is a mixture of methane and hydrogen. The cellulose equivalent of the fermented mash is about 15 grams per liter, the original dry cob concentration being about 60 grams per liter the cellulose equivalent being the calculated amount of cellulose destroyed or converted into the products mentioned above neglecting the gaseous substances.

Stirring tends to prevent the formation of methane, increases the yield of alcohol but decreases the yield of acetic acid. With stirring the same mixture as that of example 6 after seven days' fermentation yields the following:

	grams per liter
Acetic acid	8.4
Butyric acid	1.3
Alcohol	2.7
Methane	Undetermined

Butyric acid figures in this and in other examples may have a comparatively large margin of error owing to difficulty of exact ready estimation. The cellulose equivalent of the fermented products in this example is 14.7 grams per liter, the original dry concentration being 53 grams per liter.

The end products of fermentation may be varied according to the nutrient employed. Generally speaking when methane is found in the gases evolved from the direct fermentation of cellulose no alcohol is being produced at that period, but when the gases contain only hydrogen and carbon dioxide alcohol is usually produced. As an illustration of this the following may be cited:

The gas produced contained 54.2 per cent carbon dioxide and 45.8 per cent hydrogen. The products of fermentation were acetic acid in an amount equivalent to 28.7 per cent of the cellulose fermented and alcohol in an amount equivalent to 21.3 per cent of the cellulose fermented. Table 9 shows the gases produced. The kind and quantity of the products vary with the available nutrient material in the mash. The fermentation was carried out in all examples at a temperature of about 65°C.

In a mash containing the amounts of nutrient materials given in Example I no methane is produced but there is a considerable alcohol yield.

In Example II in which the amount of potassium salt is reduced to about half and a considerable amount of methane is formed, at the expense of hydrogen, analysis showed that alcohol was not produced as the final product. In Example III the quantities of the potassium and ammonium salts are maintained as in Example I but the quantity of phosphate is reduced in comparison with that example. A considerable amount of methane is again formed and a corresponding reduction in the alcohol produced was shown in the analysis.

In each case fermentation was quite vigorous and actual starvation had not set in.

The presence of methane in Examples II and III indicates clearly that while in Example I alcohol was produced as a final product, in

Examples II and III acetic acid was produced in large amounts, but little or no alcohol.

For the purpose of maintaining the hydrogen ion concentration of the mash within the limits of pH value 9.0 to 5.0, preferably 7.0, in place of sodium carbonate, sodium bicarbonate or ammonium carbonate as described above, potassium carbonate or bicarbonate may be employed or sodium, ammonium or potassium hydroxides substituted, chemically equivalent amounts being used.

In fermentation processes of the kind indicated employ a concentration of cellulose matter of not less than about 5 per cent and so to arrange the conditions of fermentation that the time in which good yields are obtained is about five to seven days.

In connection with inoculation most naturally-occurring cellulosic materials have a suitable bacterial flora already present on them and

TABLE 9

How Gases Are Produced

EXAMPLE	POTASSIUM CHLORIDE	SODIUM PHOSPHATE	AMMONIUM CHLORIDE	TYPICAL GASES EVOLVED		
				CO_2	H_2	CH_4
	grams	*grams*	*grams*			
I	0.09	0.20	0.10	56.3	43.7	—
II	0.04	0.20	0.10	62.0	9.9	28.1
III	0.09	0.05	0.10	57.2	30.4	12.4

it is not essential to inoculate such material unless in their preparation they have been subjected to a process which sterilizes them.

If desired, mixtures of green cellulosic materials and dried cellulosic materials may be employed, for example, green bamboo of fresh grass and sulphite pulp or dry corn cobs, the proportion between the two classes of materials, for instance, being such that the combined mass can be directly fermented without further inoculation.

Khouvine found that the rate of cellulose fermentation by her clostridia was increased about five times in the presence of other bacteria (6).

Dubois (7) studied the influence of environmental factors on the action of aerobic bacteria. An abundant supply of nitrogen in a buffered neutral medium with about 50 per cent moisture is optimal for aerobic fermentation at 28°C. The amount of cellulose fermenta-

tion can be determined by direct examination, the number of cellulose fermenters, the rate of carbon dioxide evolution and a chemical determination of the cellulose residue.

MacFayden and Blaxall (8) made a general study of thermophilic cellulose fermenters with a maximum range of 74°C. Their action resulted in a complete disintegration of filter paper, fibrous cellulose and esparta cellulose probably, to carbon dioxide and methane. The thermophilic cellulose destroying bacteria are widely distributed in the soil and are extremely active in the destruction of cellulose.

Viljoen, Fred and Peterson describe (4) a thermophilic cellulose fermenter, *Clostridium thermocellum*, with an optimal temperature range from 55° to 65°C. The products from cellulosic fermentation are acetic acid, ethyl alcohol, carbon dioxide, hydrogen and traces of butyric acid. Organic nitrogen is necessary in the fermentation. The organism is a facultative, sporeforming rod, 5.0μ by 0.4μ, with peritrichous flagella, Gram negative and grows well on the ordinary culture media. It stains well with carbol fuchsin, but poorly with methylene blue.

Skinner (19) has compared the relative cellulose fermenting ability of the genus Cellumonas (9) with the genus Cytophaga (18) and *Microspira agar-liquefaciens*. All of these organisms retain their cellulose fermenting power over a period of years.

A process for the manufacture of cellulose by fermentation of green plant material was patented by Loomis in 1919 (10). The object of the process is to extract the saccharine matter from the plant tissue by macerating the material in water at less than 60°C. for about twelve hours. The extracted matter may be evaporated and used as a stock feed. The residual mass with some additional nutrients will support bacterial growth of desirable aerobic pectin fermenters. Marsh grass, rushes, palmetto, corn stalks, sugar cane, sunflower stalks and banana skins, are suitable green plant material for reducing to paper and rayon pulp.

LIGNOCELLULOSE

Lignocellulose is a term applied to pure cellulose that has been changed by combination with gums and resins and is not attached by any one group of bacteria. The lignocellulose combination must first be broken down before cellulose decomposition begins. This emphasizes the importance of non-cellulose fermenters which are able to bring about this change.

In a medium containing organic nitrogen as ordinary nutrient peptone broth the colon-aerogenes group are able to reduce the lignocellulose tissue (bastose, vasculose) to intermediate products that are subject to further bacterial attack. The thermophiles that occur in feces also have this property.

WOOD

The deterioration of wood and paper pulp is an extremely important item in our economic life. When we realize that infected wood and pulp loses 75 per cent of its cellulose, pentose and other constituents through the action of microbiological agencies great loss, if not waste, is apparent. Chemical analysis of the wood before and after it is acted upon, according to Acree (11), shows that the same kind of changes are produced by pure cultures of fungi under laboratory conditions as are encountered in stored stacks of wood. Strength tests on paper made from infected pulp have less than 25 per cent of the original strength.

The organisms responsible for the rotting of wood belong to the group of higher fungi, however, it is generally noted that the presence of saprophytic bacteria increase the rate of decay (12). Such bacteria belong to the genera, Bacillus, Escherichia, Serratia, Azotobacter, etc. The type organisms are *Bacillus mycoides*, *Bacillus subtilis*, *Escherichia coli*, *Bacillus prodigiosus* and *Azotobacter chroococcum*. The best prevention against biological decay is a well built and completely aerated stack with the logs less than 6 feet long and the bark completely removed so as to facilitate drying.

HUMUS

The decomposition of vegetable débris by the action of bacteria, fungi and actinomyces results in the formation of humus, a substance made of synthetic products elaborated in the process of decomposition. There is no clear cut example as to the various steps in the process of its formation. Hebert (13) reported that during fermentation the lignocellulose, vasculose or bastose, became dissolved in the alkaline liquors of the heap to form humic substances.

In green plants the C:N ratio is about 25–40 to 1, while in humus the C:N ratio is about 10–15 to 1. The further the decay proceeds the lower the C:N ratio (14).

Dupont observed that in the anerobic portion of a manure pile

carbon dioxide, nitrogen, hydrogen and oxygen were produced (15). Deherain found the rise in temperature due to the oxygen (16).

For a complete method of analysis of natural or decomposing plant material see reference (17).

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CHAPTER 17

Textiles, Cotton, Hemp, Jute, Burlap, Retting, and Indigo

TEXTILES

The deterioration and discoloration of textiles is generally referred to as mildew (42). In cotton goods the bleaching and mercerizing processes destroy the original microflora so that subsequent contamination is responsible for actual deterioration. Here again the molds will grow on a fabric with a much lower moisture content than will the bacteria. The detrimental action of these microörganisms is observed in tent, sail and aeroplane fabric (1).

The canvas destroying fungi are:

Macrosporium	Helminthosporium
Stemphylium	Oospora
Septoria	Torulae
Alternaria	

In sampling mildewed fabric by placing a small portion in sterile tap water and plating on wort agar or Sabourand's medium an excellent growth is observed. Sabourand's medium is made as follows:

Maltose	40 grams
Peptone	10 grams
Water	1000 cc.
Agar	18 grams
pH value	5.5

The medium is sterilized in the autoclave.

The organisms associated with deterioration in textile materials consist of bacteria, yeasts and molds. The molds are Penicillia, Aspergilli and Dermatia and likely others. The bacteria include the genera *Bacillus aerobacter*, *B. achromobacter*, *B. proteus* and *B. escherichia* and likely others. All of them grow well on artificial culture media such as plain nutrient agar, litmus milk, gelatin, nutrient broth and starch agar.

DISCOLORATION

The mildew staining bacteria can be readily demonstrated when grown on the following medium of Czapek:

	grams
Sodium nitrate	2.00
Monopotassium phosphate	1.00
Potassium chloride	0.50
Magnesium chloride	0.50
Ferrous sulphate	0.01
Sucrose	30.00
Agar	15.00
Distilled water	1000.00 cc.

pH value is unadjusted.

The medium is tubed as for a stab culture, the butt being about 3 inches deep. The colonies may be isolated on ordinary culture media and then fished from the petri dish and the stab culture prepared. As the above medium is clear and colorless the brownish stain is readily detected by discoloration and diffusion into the surrounding agar.

Fabrics with 40 to 50 per cent size are most frequently subject to mildew although the amount of starch hydrolyzed is the important factor. Wheat flour is more subject to mildew when used for sizing on fabrics than is rice, cassava, potato (farina), sago or tapioca in the order named (2).

The wheat flour is frequently allowed to undergo a fermentation in preparation for use as a sizing material. The starch granules in water undergo an alcoholic fermentation with considerable frothing (3). The proteins of the wheat are rendered soluble while the sugars and part of the starch are destroyed with the production of acetic, lactic and butyric acids, ethyl alcohol and dextrins. If the fermentation becomes undesirable the vats may be cleaned with zinc chloride solution. Wheat flour so treated enables the yarn to take up as much as 30 per cent size.

Bright (4) and others showed that sizings furnish suitable nutrients for microbial growth. The only way to prevent loss from mildew is to prevent long storage, provide adequate ventilation and insure thorough drying.

Treatment with soft soap followed by 1 per cent alum and 1 per cent copper sulphate produce some retarding effect on the microbial growth. Other antiseptics that may be added to the sizings are zinc chloride, sodium silicofluoride, boric acid, salicylic acid, phenol and

turpentine. Gum tragasol, a carbohydrate from the cotyledons of the carob bean, is reported as resisting mildew. A recent patent (40) uses thorium salts.

MANILA HEMP

The bacteriology of manila hemp as reported by Prescott (5) consists of large spore forming rods and a few molds. The bacteria grow well on artificial media. As may be expected the best grades of hemp have lower bacterial counts while the poorer grades are much higher. A decrease in the microflora was observed during storage, however during six weeks incubation a definite increase was observed. Most all of the organisms were pectin fermenters. Some molds will develop on the moist fiber. The normal moisture content of the fiber is about 10 per cent.

JUTE AND BURLAP

The bacteriology of jute varies with the grade under examination. The better grade has a bacterial count of less than 50,000 bacteria per gram, whereas jute cuttings often exceed 100,000 bacteria per gram. Few molds are observed except on the moist fibers. The normal moisture content of the fiber is about 10 per cent.

The bacteria are ordinary soil and air type organisms that grow luxuriantly on ordinary culture media. Some thermophiles are observed and most of the bacteria are able to ferment pectin. Some aerobic and anaerobic cellulose fermenters are found on jute cuttings.

"Sea" and "heart" damage in baled jute is seldom observed in this country although Schepmann has observed and isolated cellulose fermenters from damaged jute received in European ports. Cross and Bevan (6) studied the chemical changes involved in typical damaged bales. They concluded that the damage was due to bacterial action. Unfortunately the rate of hemp and jute deterioration cannot be determined by the swelling test of Fleming and Thaysen. Kayser and Delaval isolated pectin fermenters from a number of different fibers.

PECTIN FERMENTATION

Pectin fermentation is produced by aerobic and anaerobic organisms. In relation to textile fibers the process is referred to as retting and consists in the separation of the cortex from the woody core and the fibro bundles from the non-fibrous portions of the cortex, by

hydrolyzing the pectin in the interstitial lamellae. Two types of retting are dew retting and steeping or water retting. The retting proceeds from the cambium layer to the outside (7).

THE PRODUCTION OF PECTINASE

Pectinase is an enzyme which breaks up the pectin nucleus to yield simple products. Pectin is a rather loosely used term signifying one of a group of substances widely distributed in nature. Protopectin, sometimes called pectinogen, is the intercellular material of all plants (8). When this material is dissolved, the cells are free and separate, so that fiber cells having a value in the textile industries are left in a usable condition.

Protopectin is apparently a mixed calcium and magnesium salt of an acid, according to Doree. Boiling water, or salts yielding an insoluble calcium compound, will liberate this acid to give the material known in commerce as pectin. This colloid is a complex anhydro-arabina-galactose-methoxyl-tetragalacturonic acid, according to Erlich, as quoted by Doree (9). It is coagulated by an enzyme, pectase, and hydrolyzed by the enzyme pectinase to form galactose, *d*-galacturonic acid, arabinose, and a methyl pentose (10). Pectinase can also act directly on protopectin to form the same compounds.

OCCURRENCE OF PECTINASE

The enzyme pectinase is found in nature in pollen, in seeds, particularly at germination, and in ripe fruit (11). It is formed by many bacteria and fungi, of which the following is a list of all those found in a search of the literature:

Bacillus amylobacter, B. subtilis, B. mesentericus..... (12)
Bacillus mycoides, B. megatherium..... (13)
Clostridium butyricus..... (13)
Erwinia melonis..... (14)
Erwinia carotovorus..... (15)
Vibrio albo-atrium..... (16)
Bacillus felsineus..... (17)
Eberthella typhi, Escherichia coli..... (11)
Fusarium cromophthoron..... (18)
Sclerotina cinera..... (19)
Mucor rasemos..... (20)
Rhizopus triciti, and all Rhizopus species, save *nigricans*..... (21)
Tyothryx kayser..... (22)

Botrytis cinera..... (11)
Saprolegmal species..... (11)
Cladosporium, Cytospora, Pythium, Colletotrichum species.... (16)
Pennicillium glaucum..... (16)

The Aspergilli do not form pectinase, according to Thom (23), nor do the various yeasts, according to Pitman and Cruess (16). Coles (24) reports that only soil organisms can attack pectin.

APPLICATIONS

Industrially the ability of certain organisms to attack pectin is most widely used in the processes known as retting. Flax, jute and hemp are prepared for textile uses by various means involving bacterial and mold action on the pectin of their bast, or fiber coats. In the classical method of flax retting, the bast fiber layers are stripped from the stalks after they have been under water, where anaerobic organisms, such as *Granulobacter pectinovorum* (25), act on the pectin to liberate the complex cellulose fiber.

A method of retting in a special tank containing the fibrous material compressed in bundles and subject to water under pressure with or without the addition of bacteria was patented (26) in 1915. As the retting proceeds fresh nutrients are added until complete dissolution is effected. Specific pectin ferments may hasten the propagation of the retting microflora. Recent improvements on this process use facultative organisms, such as *Bacillus felsineus* (17) under conditions more carefully controlled to obtain complete retting in fifty-five to seventy-five hours. The process uses the chosen organism as a predominating culture, being inoculated by the use of a starter (27).

A process for the preparation of a starter for the maceration of vegetable tissue was patented (28) by Carbone in 1923. Cultures of *Bacillus felsineus*, are added to sliced potatoes in water and the mass allowed to ferment until decomposition is evidenced. The maceration is used for inoculation of large scale fermentations. This organism is a rod, occurring singly, in pairs and in chains, motile by means of peritrichous flagella, oval terminal spores, Gram positive and with volutin granules present. Gelatin is liquefied, starch and cellulose are hydrolyzed and pectinase is formed. It is an anaerobe with an optimum temperature of 30°C. The organism was first described in the retting of flax. Doree attributes (29) the fermentation to aerobic spore forming bacteria and Kayser and Delaval (30) found aerobes

producing the retting in forty-eight hours at 25°C. However, aerobic conditions are not necessary in all cases.

In the dew method of retting, the piles of bast are spread on upland downs, over the natural plants of the region. Here the dews keep the bast moist, and molds naturally on the fibers do the retting in from two to five days, according to Thaysen and Bunker (31). It has recently been suggested that the milkweed would make a valuable addition to the list of textile fibers if retted in similar ways (32). Waksman states (11) that if an enzyme preparation of suitable strength could be developed, the retting of vegetable fibers could be carried out more rapidly and would yield a better and more uniform product.

Flax is approximately 7 per cent pectin, according to Kind (33), as it enters the retting tank, and the retting of hemp is said by Schafer and Simmonds (34) to cost about three cents per pound, or just about the same as the cost of preparing and growing the bast. A diastatic enzyme preparation of sufficient activity to digest 40 to 200 times its weight of starch is thought to be of sufficient value to be marketed, according to a recent patent (35). Using these figures as bases for a very rough calculation, it is seen that a pectinase for retting hemp would have to sell for about eleven dollars per pound to compete with the natural retting processes. This assumes that the enzyme would dissolve forty times its weight of pectin, or rett 560 times its weight of fiber. It also assumes that handling and interest charges on equipment would not cost more than one cent per pound of hemp. This figure is not so low as to be discouraging, especially when it is remembered that flax is a more valuable fiber and would probably afford a more expensive enzyme.

Marcus (41) has patented a specific retting organism for the pectin which he describes as a nitrogen fixing bacterium. The process of retting consists in agitating the fiber for about fifteen minutes in water, inoculated with the microörganism, at about 40°C. followed by allowing the fiber to remain immersed for approximately three hours at the same temperature.

There is also a commercial process for preparing starch by dissolving the pectin surrounding the grains by bacterial action, as described by Thaysen and Bunker (31). An enzyme could be used to advantage here, because the bacteria consume about 15 per cent of the starch they prepare.

INDIGO

The action in the cold oriental indigo vat is due to a bacterial fermentation by a slender rod. A patent was taken out in 1917 (36) to cover a method of preparing a mixture of albumin extracts and invert sugars which when added to the cold lime fermentation vat will facilitate fermentation.

The fermentation method is now superseded by synthetic methods using the Baeyer process.

COTTON

Cotton is subject to the action of bacteria when the moisture content is over 9 per cent. The amount of destruction can be determined by the method of Fleming and Thaysen (39). The damage is directly due to the microörganisms that grow in the fibers. The action of sea water on fibers is still questionable although the resulting deterioration is the same regardless of the source of the moisture. Doree (37) found that sea water, as such, has no effect on the cotton fiber if bacterial growth is prevented.

Fleming and Thaysen (38) found that cotton with a count of 1,400,000 organisms per gram when kept at 16°C. for three days remained unchanged, when the moisture content was 6.5 per cent. In the same period of time the presence of 10 per cent moisture in the cotton gave counts of 124,000,000 organisms per gram, 20 per cent moisture gave counts of 1,112,000,000 organisms per gram, 30 per cent moisture gave counts of 1,500,000,000 organisms per gram and 50 per cent moisture gave a count of 9,040,000,000 organisms per gram. The same investigators report that for American seed cotton exposed to little or no rain, 220,000 bacteria per gram, 4000 molds per gram and 8.9 per cent moisture; cotton after ginning 1,200,000 bacteria per gram, 1000 molds per gram and 6.9 per cent moisture; cotton after pressing 7,000,000 bacteria per gram, 20,000 molds per gram and 7.3 per cent moisture. Thermophiles were present in all three samples. The mesophiles were all typical soil forms. Attention is called to the usual storage temperature of 15° to 30°C. which serves for incubation of the flora. Several cellulose fermenters were found on some of the samples.

Cotton can very easily attain sufficient moisture to sustain bacterial and mold growth. Bacteria require a minimum of 9 per cent moisture whereas molds require a minimum of 7 per cent mois-

ture. In the textile industry it is customary to calculate on the basis of the dry weight.

According to Schloessing's curves raw cotton at 12°C. and 90 per cent relative humidity will have a moisture content of about 15 per cent.

Microörganisms affecting the cotton plant belong to the following genera:

Erwinia—Motile rods, possessing peritrichous flagella, Gram negative, aerobic and facultative.

Phytomonas—Rods, motility varies, aerobic and facultative, Gram negative.

To these genera could be added the numerous cellulose fermenting bacteria which are important in subsequent contamination of the cotton fibers and the attendant deterioration.

Thaysen and Bunker (43) have definitely demonstrated in a series of experiments that *Bacillus subtilis* and *Bacillus mesentericus* are not capable of decomposing cellulose. These organisms are frequently isolated from deteriorated cotton goods but they derive their nutrient from the size.

It is noted that practically all the types of microörganisms found on cotton samples and textiles generally, are representative of the subtilis-mesentericus group. This group produces deterioration of the cotton fabric by production of brownish or grayish stains as is evidenced on damp material when allowed to remain relatively short periods of time, in a warm place. The ability of these organisms to produce such stains can be demonstrated on Czapek's agar as referred to in the discussion of mildew on textiles.

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CHAPTER 18

Ensilage Fermentation

Ensilage affords a means of preserving valuable food for stock by encouraging desirable fermentation, either enzymatic or microbial. Babcock and Russel (1) believe it to be a respiratory process of the plant tissues. Esten and Mason (2) refer to it as the product of bacterial and plant tissue enzymes, while Sherman (3) stresses the importance of the genus, Lactobacillus. Hunter and Bushnell (4) found a *Lactobacillus bulgaricus* type predominating in the microflora, in all stages of the fermentation, with silage prepared from cane, kaffir, corn, alfalfa and alfalfa with different kinds of carbohydrate material. The number of bacteria correlate with the acidity of the fermentation.

Heineman and Hixson (5) observed three phases in the fermentation process. The first or acid and gas phase is produced by the colon-aerogenes group, the second or acid phase is produced by the streptococcus-lactobacillus group and the third phase is definitely acid with many streptobacilli predominating. A temperature slightly above 35°C., is optimal for this genus and the lactic enzymes.

The genus Lactobacillus is described as follows:

> *Lactobacilli*—Long slender rods, Gram positive, non-motile, non-spore formers, microaerophilic and facultative anaerobes.
>
> Generally produce lactic acid from carbohydrates, the optical activity depending upon the species producing the acid. Carbon dioxide and hydrogen gas may be formed.

According to Breed, *Bacterium acidi-lactici* belongs to the genus Escherichia. This organism produces acid and gas and is found in the intestinal tract and in the soil.

The surface of the plant tissues used in silage production contains many bacteria varying from 10,000 to as high as 400,000,000 bacteria per gram. The silage juice may have a bacterial count of from 2,000,000,000 to 4,000,000,000 bacteria per cubic centimeter. During the fermentation lactic, acetic and butyric acids are produced, but vary widely in their respective percentages, depending upon the fermented material. Vegetable tissue that has become heavily con-

taminated with soil is likely to produce butyric acid predominantly, with putrefaction and other undesirable changes. Gorini (6) found butyric acid in his fermentations. A small amount of alcohol is produced during the fermentation and carbon dioxide values up to 80 per cent are found.

Hunter has demonstrated (7) that heat production in forage fermentation results from microbial activity and not from intramolecular respiration of the tissue cells. Loosely packed silage shows a rise in temperature at the top of the silo where air has access to the fermenting mass. Green fodder with about 70 per cent moisture will undergo heating when packed loosely in aerated layers. A process for preserving green fodder taking advantage of this occurrence was patented in 1918 (8). As the fodder ferments with a rise in temperature to about 60°C. a new layer is thrown on in a loose manner. When the second layer reaches about 50°C. additional layers are placed above the heating portions, allowing such a temperature to be attained each time a new layer is added. When the silo is full and the top layer reaches about 55°C., pressure is applied. This compresses the fermenting mass, excludes the oxygen and reduces the heat production.

Fermentation is enhanced by inoculation of each successive layer with whey cultures of *Escherichi acidi-lactici* incubated at 38°C. for about thirty-six hours. Approximately 100 cc. of culture is required per cubic yard of fodder. The by-product whey from cheese manufacture affords an excellent culture medium after the albumin is removed by boiling.

Wyant (9) inoculated corn silage to hasten fermentation. Russel (10) found fermentation proceeding with Lactobacilli as the predominating group. All of these organisms will grow on the ordinary media and are easily transferable.

Hastings and Mansfield (11) found a small aerobic rod responsible for the rotting of silage. When the organism was added to sterilized silage it produced a reduction in the acidity with the production of an abnormal odor. The organism is acid tolerant and salt tolerant up to 2.5 per cent.

Owen and Bennett (12) found an aerobic organism responsible for the fermentation of bagasse.

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CHAPTER 19

Xylose Fermentation

INTRODUCTION

From a historical review of the literature it is apparent that little attention had been paid to the bacterial fermentation of xylose before the entrance of the United States into the World War. Stone (5), Fischer and Lafar (30), and others, found that yeasts alone do not ferment xylose. These findings were confirmed by Cross and Tollens (29). In addition Cross and Tollens found that yeasts in a nutrient culture medium assimulated a small amount of xylose, also that the bacteria commonly found in pressed yeasts had the power to ferment xylose with the production of lactic acid.

The occurrence of xylose fermenting bacteria in the feces of man and cows was reported by Bendix (20) in 1900. Friedlander's bacillus is claimed by Grimbert (17) to transform xylose into alcohol, succinic and acetic acids, also traces of lactic acid. Kayser (15), in his work on sauerkraut isolated an organism from the aforementioned food product which fermented a number of sugars including the pentoses arabinose and xylose. The acid produced by these sugars was lactic, however, both lactic and acetic acid was obtained by the fermentation of dextrose by the same organisms.

Lohnis (25), and Stoklasa (26), indicate that xylose as a source of carbon for the nitrogen fixing bacteria is superior to the disaccharides lactose, maltose, sucrose and many other sugars. Emmerling (18) reports *Bacillus mycoides* as a fermenter of xylose and arabinose with the formation of small quantities of non-volatile acids. According to Salkowski (21), protein-decomposing bacteria in a rich nitrogenous medium break up xylose with the production of a volatile acid, acetic, and a non-volatile acid, succinic.

The intestinal bacteria of the guinea pig ferment xylan rapidly and produce notable quantities of fatty acids consisting of about eight parts of acetic acid to one of butyric acid (28). McCollum and Brannon (27) report that the pentosans of the corn plant disappear more rapidly in the digestive tract of the cow than those of wheat and oat plants, the amounts digested exceeding 50 per cent.

Foote, Peterson and Fred (59) claim the fermentation of a typical hexose, glucose and typical pentose, xylose by four species of nodule bacteria, *Rhizobium meliloti*, *Rhizobium trifoli*, *Rhizobium leguminosarum* and *Rhizobium japonicum* in yeast water carbohydrate media. Fred, Peterson and Davenport (36, 39) call attention to the occurrence of certain pentose fermenters in soil, manure, sauerkraut and silage. Allgeier, Peterson and Fred (58), report the production of acetic and lactic acids from mill sawdust after hydrolysis by bacterial fermentation. The pentoses, xylose and arabinose are rapidly and completely broken down to acetic acid, lactic acid and only the merest trace of other products such as carbon dioxide and alcohol, has been reported by Fred, Peterson and Davenport (39).

From this short review of the literature on fermentation it is obvious that the information is brief and at times uncertain, especially is this true up until 1915. During the aeroplane development program at the time of the World War it was quite evident that chemical producing industries were confronted with many serious problems of shortage. In the compounding of aeroplane "dopes," acetic acid and its derivatives, amongst which may be acetic anhydride, acetone, methyl acetate, amyl acetate, were necessary as solvents in the manufacture of cellulose acetate which was used as "dope." The manufacture of smokeless powder had absorbed practically all the acetone produced; as a result, the Department of Science and Research, Bureau of Aircraft Production, Chemical Section, in connection with the Bureau of Chemistry, undertook many lines of research, one of which led to the bacterial fermentation of xylose.

It was first suggested to H. D. Gibbs, of the Bureau of Aircraft Production, by L. A. Round, of the Bureau of Chemistry, "that xylose might be fermented into some valuable compounds." A method for the production of xylose on a commercial basis at a low cost was demonstrated by Hudson and Harding. Recently semi-commercial production of xylose has been carried out by Schreiber, Geib, Wingfield and Acree (62). At the suggestion of H. D. Gibbs the bacteriological work was attempted by E. B. Fred of Wisconsin University. Round assisted Fred by furnishing him with the information he had already gathered and also supplied a culture that he had isolated. The xylose was furnished by the carbohydrate laboratory.

With this aid we learn that Fred and Peterson have obtained several patents (45a, 57) on the fermentation of xylose by *Lactobacillus pentoaceticus* and another closely related organism.

Their xylose fermenters were isolated by plating in xylose yeast agar made from yeast water, the preparation of which is described later. The organisms were found in silage, sauerkraut and manure. *Lactobacillus pentoaceticus* was the first to be isolated. It was found to produce two to three times as much acid from xylose as from fructose, glucose, sucrose, lactose or mannitol (36).

Fred, Peterson and Anderson (45) have recognized and described four new species of xylose fermenting lactobacilli on the basis of action on milk and of carbohydrate fermentation as shown in table 10.

The morphological description given later of the last will suffice for all except for minor differences in size. They range from 1.6 by 0.6 microns to 3 by 0.7 microns in size. Their optimum growth tem-

TABLE 10

FOUR NEW SPECIES OF XYLOSE FERMENTING LACTOBACILLI

	ARABINOSE	XYLOSE	LACTOSE	DULCITOL	MELIZITOSE	FRUCTOSE
I. Coagulate milk slowly; do not form mannitol from fructose						
Lactobacillus pentosus A	+	+	+	0	0	+
Lactobacillus pentosus B	+	+	+	+	0	+
Lactobacillus arabinosus	+	0	+	0	+	+
II. No coagulation in milk; form mannitol from fructose						
Lactobacillus pentoaceticus	+	+	0	0	0	+

perature is 30° to 35°C. and their pH range for growth is a value 2.8 to 9 with an optimum value of 5.6. Volatile acid produced from pentoses is almost pure acetic and fixed acid is racemic lactic.

$$\underset{150}{C_5H_{10}O_5} = \underset{60}{C_2H_4O_2} + \underset{90}{C_3H_6O_3}$$

or 40 per cent acetic to 60 per cent lactic. Anaerobically there is from 90 to 94 per cent conversion of sugar to acids and aerobically 84 to 92 per cent. Maximum acid yields are found in fourteen to sixteen days.

DISTRIBUTION OF PENTOSES

Xylose ($C_5H_{10}O_5$) is a pentose sugar present in many plants as polyoses termed pentosans (47). The sugar does not appear in the

free state in nature, but is widely distributed in the form of a condensation product, gum xylan (34). It has been obtained from brewers grain (2), straw (6), corn cobs (7), jute (3), hay (11), and flax (14), the alkaline cook liquor obtained by the action of hot calcium hydroxide on straw in paper making (14), cocoanut shells (16), certain tragacanth gums (22), apricot stones (23), *Psyllium gallicum* or "fleawort," a species of plantain found in southern Europe (1), sawdust (56), peanut shells, and may be obtained from any substance displaying the reaction of lignin (31). Lignin gives a yellow coloration with salts of aniline and a red coloration with a solution of phloroglucinol (47).

We know, however, that the substance changes the secondary and tertiary cell walls in plants. This change brought about by the impregnation of pectin and cellulose is spoken of as lignification. Lignification results in the strengthening of the fibers. Possibly the best example is that of the lignification of cells termed stone cells, which make up the greater portion of the shell surrounding an English walnut or better still an apricot stone. This later material has been used for the production of xylose (23).

HYDROLYSIS OF WOOD

Fred and Peterson in their patent (45a) state that hydrolysis was accomplished with dilute acids under suitable conditions of temperature and pressure, as 2 per cent sulphuric acid at a temperature of 15 to 20 pounds steam pressure for one to two hours. Filtering off the unhydrolyzed material results in a clear amber colored liquid containing pentose sugars. In order to bring about a fermentation of the above liquid it is necessary that the acid solution be first neutralized. They suggest neutralization with milk of lime or other weak alkali or potential alkali in suspension or solution. Calcium carbonate makes an excellent neutralization agent. Buffering with calcium carbonate at the beginning gives equally as good results as periodic neutralization with sodium hydroxide. In another patent (57) the same investigators suggest hydrolysis with 2.5 per cent sulphuric acid at a temperature of 100 pounds steam pressure for fifteen minutes.

DESCRIPTION OF ORGANISM

Lactobacillus pentoaceticus, Peterson and Fred. Rods, 0.6 to 0.7 by 1.6 to 2.5 microns with blunt ends, occurring singly and in short chains, occasionally as filaments. Gram positive.

Gelatine stab: Growth uniform along line of inoculation. No liquefaction.
Agar colonies: Small, grayish, smooth, entire.
Agar slant: Scant, bladed, glistening.
Broth: Slowly becoming turbid.
Litmus milk: Unchanged.
Potato: No growth.
Indol not formed.
Nitrates not reduced.

The aldehoses are fermented with the formation of lactic acid, ethyl alcohol, carbon dioxide and small quantities of acetic acid. Dextrose and galactose are fermented at approximately the same rate, mannose less actively. Ethyl alcohol is one of the principal products in the fermentation of dextrose while acetic acid is one of the principal products in the fermentation of fructose and xylose.

Microaerophilic.

Optimum temperature, 30° to 35°C.

Habitat: Soil, manure, ensilage, sauerkraut.

CULTURE MEDIA

According to Fred, Peterson and Davenport (36), all attempts to grow the pentose fermenters in synthetic media failed. They tried urea, asparagin and ammonium sulphate.

After some experimentation with the above media, they conceived the idea of using yeast water. Since it was impossible to grow the organisms in a synthetic medium, free from other sources of carbon, which is desirable in determining the value of substances as a source of carbon. It was found advisable to run controls on the yeast water alone plus the organism and subtract the results of the control. Yeast water alone contains a very small amount of available carbon compounds.

The yeast water as used by the above experimenters was prepared as follows: 450 grams of pressed yeast were steamed three to four hours wth 4500 cc. of tap water with occasional stirring. This infusion was then allowed to stand undisturbed until the heavy deposit of yeast cells and other suspended matter had settled to the bottom of the vessel. The portion above this deposit was carefully poured off and sterilized in liter flasks. These flasks of the sterile yeast infusion were allowed to stand one to two weeks, when the supernatant liquid was syphoned off and used without clearing or filtering.

For a few of the experiments this medium was cleared with egg

albumin and a clear yellow liquid resulted. Two per cent of carbohydrate was generally added, and if calcium carbonate was not added or to be used the reaction of the medium was adjusted to a pH of about 7.0 with phenol red. The resulting adjusted medium was accurately measured and pipetted into Erlenmeyer flasks and sterilized at 12 pounds for thirty minutes.

The settling of the yeast cells for two weeks has been found unnecessary in the writer's laboratory. After allowing the greater portion of cells to settle, which generally takes two to five days depending upon the size of container, the supernatant liquid is decanted and heated to almost the boiling point, then filtered through Keiselguhr (diatomaceous earth), or better still a Berkefeld filter. The filter used in the work thus far has been the Mandler filter. Yeast water thus prepared gives a pH value between 7.6 and 7.8 which may readily be adjusted to the desired hydrogen ion concentration.

W. Heintzelman, while working with the senior author conceived the idea that alfalfa leaves might be a source of nitrogen for the growth of *Lactobacillus pentoaceticus*. It is highly probable that the leaf is the seat of protein synthesis, as it is already known to be the seat of carbohydrate synthesis. The close relations of amino-acids to carbohydrates and the known conversion of ketonic acids into amino-acids suggest chemical possibilities which deserve consideration. From this point of view the protein of the leaf may represent the original protein from which all other kinds are formed, either directly or indirectly.

Alfalfa broth infusion was made by autoclaving 1000 grams of alfalfa leaves in 10 liters of water for fifteen minutes at 15 pounds of steam pressure. At the end of the extraction period the resulting liquor was strained and then filtered through a Mandler filter. The resultant medium had a pH value of 5.6.

EFFECT OF COLLOIDS AND PHOSPHATES

The simulating effect of colloids has been reported by Grieg-Smith (46), (48), (52). Allgeier, Peterson and Fred (58) state that fermentation of sugar liquid obtained by hydrolysis with sulphuric or hydrochloric acid (Rheinau and Prodor processes) (49) was not accelerated. Malt sprouts (5 per cent) were added to the diluted neutralized sugar liquor as a source of nitrogen and remained partially suspended, therefore the addition of more colloidal substances has no effect. The additions were willow, charcoal, talc, and fuller's earth.

Vertanen (53) states that phosphates produce a favorable influence upon certain lactic fermentations. Malt sprouts should again fill the bill as a phosphate supply as they are known to contain the salts. Malt sprouts are of interest commercially in this problem as they are a source for the cheapest form of nitrogen that can be utilized by the organisms concerned.

DETERMINATION OF END PRODUCTS

Fred and others (36) determined the unfermented xylose in cultures by the O'Sullivan method. They did not determine the reducing ratio of glucose and xylose by this method. The value used was calculated from the papers of Brown, Morris, and Millar (19), and Daish (31) in which the method is similar to the O'Sullivan-Deferns method, giving results almost identical with that of Brown, who found the ratio to be 0.983 with the Allihn method.

Their later work indicates that they are using a modification of the Schaffer-Hartman method (41). This method is suited for the determination of arabinose, xylose, galactose, fructose, maltose, lactose and sucrose after inversion by applying the necessary factor for the sugar determined. The method is known as Stiles, Peterson, and Fred modification (50) of the Schaffer-Hartman method.

The volatile acids were determined by the Duclaux method after distilling the entire culture, approximately 50 cc., with 20 grams of acid sodium phosphate and 15 cc. of 85 per cent phosphoric acid (36). During the distillation the volume of the liquid must be kept constant (32). This is accomplished by adding water from a drop funnel. Distillation is carried on over a low flame for about two hours until 150 to 200 cc. of liquid is driven over, or until the fresh distillate gives no acid reaction to litmus.

Lactic acid may be tested qualitatively by means of the Uffelman and the thiophene tests. Quantitatively it is determined by means of its salts as lactates of barium and zinc. Zinc lactate makes it possible to ascertain the form of acid produced. Lactic acid exists in three stereoisomeres (47). Carbon dioxide may be determined by means of Van Slyke's apparatus for determining CO_2 in blood and carbonate solutions.

PRODUCTIONS OF ACETIC AND LACTIC ACIDS FROM MILL SAWDUST

Allgeirer, Peterson and Fred (58) hydrolyzed commercial mill sawdust (fir, spruce and pine) with dilute sulphuric acid and the resulting sugar liquors were fermented with a lactic acid organism which ferments both pentoses and hexoses. Malt sprouts (5 per cent) as a source of nitrogen and excess of calcium carbonate as a neutralizing agent must be added to the sugar liquor in order to bring about a satisfactory fermentation. As inoculum for small batches of sugar solution use 2 per cent of a twenty-four hour yeast water culture, and for larger batches 5 per cent.

Although 2 per cent sugar concentration gives best results with recently isolated cultures yet the organisms may be educated by increasing concentrations of successive fermentations to ferment up to 10 per cent sugar solutions. By this process 7 to 9.6 per cent sugar solutions may be 85 per cent converted to lactic and acetic acid in nine days. A yield of acid equivalent to 95 to 100 per cent of the sugar destroyed, is obtained. This acid consists of 90 to 95 per cent lactic and 5 to 10 per cent acetic acids.

Sugar obtained by the Bergius (56) process fermented equally as well as the sugar liquor obtained by the sulphuric acid process and gave approximately the same yield of products.

COMMERCIAL APPLICATION

If commercial yields would be the same as laboratory yields it is possible to produce 316 to 950 pounds of lactic acid per ton of sawdust, dry weight depending upon whether the sulphuric process of hydrochloric process is used. The sulphuric process yields less (see table 11).

In 1921 Fred and Peterson (42) discussed the production of acetic and lactic acids from fermentation of the hydrolyzed liquor from corn cobs.

The cobs were hydrolyzed with 2 per cent sulphuric acid for one to two hours at 15 pounds steam pressure. According to Hudson and Harding (34) this would yield a syrup containing 30 per cent adhesive gum, 5 per cent crystalline xylose, 2.5 to 3 per cent acetic acid and 37 per cent crystalline glucose, but Fred and Peterson obtained 30 to 40 per cent of a furfural yielding substance which they calculated as xylose. Of this xylose 85 to 90 per cent was converted into the two acids, 43 per cent acetic and 57 percent lactic. One ton of cobs should yield 300 pounds of acetic and 320 pounds of lactic acids.

ALCOHOL FERMENTATION PRECEDING THE ACID FERMENTATION

Sugars obtained by wood hydrolysis may be first fermented by yeasts to form alcohol thus separating quantitatively the mannose and glucose. The beer so obtained may be then filtered, concentrated under reduced pressure to one-third volume to remove alcohol, refiltered by suction with washing of the sludge and dilution of the filtrate with yeast water to a 2 per cent sugar concentration. Then add 5 per cent malt spouts and an excess of calcium carbonate, sterilize and inoculate with *Lactobacillus pentoaceticus* (44).

Peterson and Fred (38) also discuss the fermentation of fructose by *Lactobacillus pentoaceticus* with the production of malic, acetic and lactic acid, carbon dioxide and manitol. Early in the fermentation 30 to 40 per cent of the fructose was converted to manitol which later

TABLE 11

YIELDS FROM A TON OF SAWDUST (DRY WEIGHT)

	SULPHURIC PROCESS	HYDROCHLORIC PROCESS
Yield of sugar, pounds	400	1200
Sugar fermented, pounds	340	1020
Concentration lactic acid in fermented liquor, per cent	7–8	7–8
Concentration acetic acid in fermented liquor, per cent	0.35–0.4	0.35–0.4
Lactic acid, pounds	316	950
Acetic acid, pounds	17	50

was further fermented to acetic and lactic acids. All of the fructose disappears in five days, but the further manitol fermentation is slow. They give the following formulae showing the malic acid as an intermediary to lactic acid:

$$C_6H_{12}O_6 + H_2O = C_2H_4O_2 + C_4H_6O_5 + 2\ H_2$$

Acetic acid — Malic acid

$$C_4H_6O_5 = C_3H_6O_3 + CO_2$$

$$2\ C_6H_{12}O_6 + 2\ H_2 = 2\ C_6H_{14}O_6$$

Manitol

This process we do not believe has been used commercially.

OTHER XYLOSE FERMENTATIONS

Fred, Peterson and Anderson (45) state that xylose and arabinose can be fermented by *Bacillus vulgatus*, *Aerobacter xylinum*, *Aerobacter sorbose*, *Bacillus herbicola aureum* and two yellow cocci, the products depending on the kind of organisms and their ratio on the age of the culture. Acetone, ethyl alcohol, CO_2 and small amounts of fixed acid may be formed.

Breden, Fulmer, Werkman and Hixon (61) have fermented xylose as well as sucrose with *Aerobacter faeni* with the formation of formic, acetic, succinic and lactic acids, ethyl alcohol, CO_2, H_2, 2.3 butylene glycol and acetyl-methyl-carbinol. This work was as a study of the utilization of agricultural wastes, and the organism was selected for its good growth on synthetic media with ammonium sulphate. Other hexoses and related compounds can be fermented by these lactobacilli.

Glucose is 99.9 per cent destroyed to form 90 per cent lactic acid, and lactose, raffinose and melezitose almost equally so. Carbon dioxide is formed in small amount, 0.01 to 0.03 grams per gram of sugar probably not as a direct fermentation product but as a product of cell respiration (44). Small amounts of alcohol and acetic acid are formed from glucose, galactose and mannose, the latter probably as a secondary fermentation product of the lactic acid (40).

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CHAPTER 20

Microflora of Sugar, Preservation of Sugar

MICROFLORA OF SUGAR

In 1911, Owen investigated the presence of spore-forming rods in sugar to determine their relation to the deterioration during storage. In a later report (1) he attributes the loss largely to the activity of mold and yeast cells. Browne (2) isolated *Torula communis*, *Monilia nigra* and *Monilia fusca* and attributes the decomposition to their activity. Weinzirl (3) reports the isolation of colon organisms from candies. James (4) was able to isolate *Escherichia coli* in 2 out of 50 samples of raw sugar with total counts ranging from 20 organisms to 6000 organisms per gram. Putrefactive anaerobes were isolated from sugars of all types as were also the spoilage thermophils referred to in food bacteriology as "flat sour," "sulphur" and "hard swell" types. Flat sour type organisms are acid formers with no gas formation, whereas hard swell type organisms are usually acid and gas formers. The sulphur type organism is *Clostridium nigrificans*. All these organisms have an optimum temperature near 55°C.

Table 12 lists some of the carbohydrates suitable for microbial fermentation.

Preservation of Sugar

The mold fungi are the causative agencies in the deterioration of sugars. Owen patented (5) a process for the preservation of white sugar by inoculating the sugar with a yeast that has the ability of growing in the relative high density of the molasses films surrounding the sugar crystals and saturating the film with carbon dioxide gas which in turn prevents the mold growth. The yeast, Torulae, does not attack sucrose, but forms the carbon dioxide from the levulose present. The loss of levulose makes the sugar less hydroscopic and tends to reduce the color of raw sugars. The inoculating material is used in quantities approximating 5 per cent by weight, of a molasses having about 1,000,000 yeast cells per cubic centimeter. The yeast-molasses mixture is sprayed on the sugar in the proportion of 1 to 10,000 yeast-mixture to sugar.

TABLE 12

CARBOHYDRATE FERMENTATION

I. Monosaccharides:
  Pentoses:
    1. Arabinose.
    2. Ribose.
    3. Rhamnose.
    4. Xylose.
  Hexoses:
    1. Galactose.
    2. Glucose.
    3. Fructose.
    4. Mannose.
II. Disaccharides:
    1. Lactose-glucose and galactose.
    2. Maltose-glucose and glucose.
    3. Sucrose-glucose and fructose.
III. Trisaccharides:
    1. Raffinose-fructose, glucose and galactose.
IV. Colloidal Polysaccharides:
  Starches:
    1. Starch-glucose
    2. Inulin-fructose.
    3. Glycogen-glucose.
    4. Dextrins-glucose.
  Celluloses:
    1. Cellulose-glucose.
    2. Lingnocelluloses-glucose.
    3. Hemicelluloses.
      Pentosans:
        Gum arabic-arabinose.
        Straw-xylose.
      Hexosans:
        Galactan:
          Agar-agar-galactose.
        Mannans.
    4. Pectocellulose:
      Pectin-hexoses and arabinose.
      Gums-arabinose and galactose.
      Mucilages-non-reducing sugars.

The efficiency of the process depends upon the number of Torulae present in the inoculating material, the rate of growth of the yeast cells, the per cent of carbon dioxide in the inoculating material and the

per cent of carbon dioxide in the finished sugar. The increase in the polarization of sugars inoculated by means of this protective process gave a net increase of between $3.00 to $4.00 per ton in the price of raw sugar on the New York market. In addition to giving better keeping qualities and color to the sugars and reducing the affinity for moisture, the process gives increased initial polarization at practically no expense. A Cuban molasses increased in polarization eight degrees and the sugar filmed with it by this process increased 1.4 degrees in polarization. Torulae do not form ascospores but resemble the true yeast cells in morphology and reproduce by budding. They are sometimes termed wild yeasts, false yeasts, pseudo yeasts or mycodermae.

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Section V

THE FATS AND OILS

CHAPTER 21

Yeast, Mold and Bacterial Fats

FERMENTATION OF FATS

Chemically considered the fats are esters of the tri-atomic alcohol, glycerol, and the mono-basic fatty acids. In the fermentation of fats the initial phase consists in the production of fatty acids and glycerin by the microbial enzymes. This splitting or lipolysis takes place only in the presence of moisture and is further facilitated in the presence of more organic matter. A medium according to Rahn (1) is made as follows:

	grams
Dipotassium phosphate	5.0
Ammonium phosphate	5.0
Magnesium sulphate	1.0
Ferric chloride	trace
Calcium chloride	1.0
Sodium chloride	trace
Distilled water	1,000 cc.

The fat is placed in a flask containing the above solution and is inoculated with *Clostridium butyricum*, *Pseudomonas nonliquefaciens*, *Pseudononas aeroginosa*, etc., or other lipolytic organisms. Many species that are unable to attack the fat will ferment the glycerol produced and other secondary products of fermentation. *Escherichia coli* converts glycerol into alcohol and formic acid (2). While the splitting is carried out more completely under anaerobic conditions the fermentation of the fatty acids is usually produced by bacteria and mold in the presence of free oxygen.

YEAST FAT

When yeast cells are grown in well aerated or oxygenated solutions of glucose, fructose or sucrose the fat content becomes very markedly increased. The addition of alkaline phosphate to the fermenting sugar may increase the fat yield by 100 per cent. Daubney and Mac Lean (5) examined the fatty acids of yeast fat and found them to consist of oleic, linolic and palmitic, which were identified together with

possible traces of other acids, namely, lauric and arachidic. The relative amounts of these different acids can be very largely influenced by altering the constituents of the medium in which the yeast cells are growing.

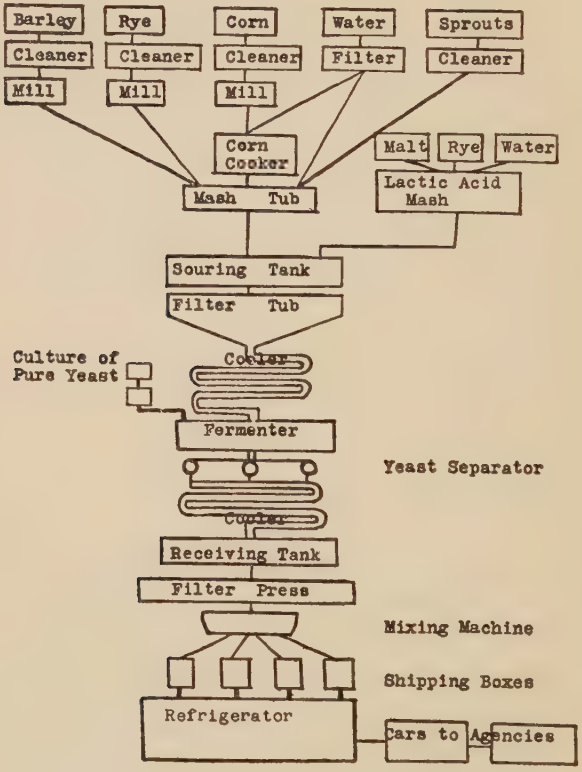

FIG. 6. DIAGRAM OF YEAST MANUFACTURE
(Courtesy of Standard Brands)

Table 13 shows the approximate composition of normal yeast cells. Yeast fat consists largely of unsaponifiable matter (as much as one-third by weight of the total fat).

MOLD FAT

Pearson and Roper (3) used two molds, *Aspergillus niger* and *Rhizopus nigricans*, to study the influence of the temperature on the

nature of the fat formed by living organisms. *Aspergillus niger* was grown on Czapek's solution to which 1 per cent ammonium sulphate and 3 per cent glucose were added. *Rhizopus nigricans* was grown

TABLE 13

Approximate Composition of Normal Yeast Cells

	DRY	WET
Protein	52.41	14.15
Fat	1.72	0.46
Glycogen	30.25	8.16
Cellulose	6.88	1.87
Ash	8.74	2.36
Water	—	73.00

TABLE 14

Influence of Temperature on Nature of Fat

TEMPERATURE	TIME OF GROWTH	MOIST WEIGHT	TOTAL WEIGHT FATTY ACIDS	MEAN MOLECULAR WEIGHT	IODINE NUMBER
		Aspergillus niger			
	days	*grams*			
18°	17	49.8	0.323	303	146.7
	56	39.9	0.084	323	150.2
25°	14	50.3	0.302	304	132.5
	35	40.5	0.252	287	127.0
35°	6	39.2	0.468	293	92.1
	9	37.8	0.633	290	99.9
		Rhizopus nigricans			
12°	30	34.6	1.705	281	88.7
	30	31.0	1.669	289	87.2
25°	10	32.5	1.082	288	89.0
	13	35.4	1.168	287	77.3

on Meyers' solution with the addition of 1 per cent asparagine and 3 per cent glucose. Sterile liter flasks containing 500 cc. portions of the medium were inoculated and incubated at 18°, 25° and 35°C., respectively, for *Aspergillus niger* and 12° and 25°C. for *Rhizopus*

nigricans. It was found impossible to grow this organism at 35°C. The flasks were incubated until a good mat had formed and covered the surface of the medium. The mat was broken up, strained and pressed on a Buchner funnel and then well washed with distilled water. After roughly drying between filter paper the mat was weighed. It was then covered with alcohol and allowed to stand twenty-four hours. The alcohol was removed by filtration and the fungus mat extracted with hot alcohol by suspending it in a thimble from the lower end of a reflux condenser which was attached to a flask containing 75 cc. of boiling absolute alcohol. This was extracted for three hours followed by extraction with a fresh 75 cc. portion. The three alcoholic extracts were combined, 15 cc. of 50 per cent potassium hydroxide added and most of the alcohol distilled off leaving the saponified fat, fatty acids and unsaponifiable matter for chemical determination. Table 14 is taken from their publication.

The following data are drawn largely from the work of Terroine, Bonnet, Kopp and Vechat (4) published in 1927. Composition of St. nigra:

	17°C.	*35°C.*
Protein	37.05	35.06
Fatty acids	10.91	12.32
Unsaponifiable	1.05	1.60
Ash	3.00	3.00
Cellulose	47.99	48.46

The iodine values of the fatty acids produced by the same mold when grown on a 30 per cent glucose solution are as follows:

36°C.	*17°C.*
83	114
87	112
86	116
Average, 85	114

The composition of a bacterial cell "*Bacillus subtilis* type" according to the same investigators is:

	14°C.	*35°C.*
Protein	36.56	34.37
Fatty acids	25.92	25.00
Unsaponifiable	5.72	9.60
Ash	3.00	3.00
Cellulose	28.80	28.03

The iodine values of the fatty acids produced by the same species of microörganism grown on a 12 per cent glucose solution are:

35°C.	14°C.
35	57
31	59
35	—
Average, 33	58

EDIBLE FATS

In 1921, Jones patented (6) a composition consisting of edible fats and oils mixed with a bacterial culture that has undergone lactic fermentation. In order to prepare a product similar to butter, oleo, margarin, butterin, oil butters, etc., without producing "off" flavors a controlled fermentation is carried out to a definite end point. Suitable media consists of:

	grams
Bran	22.5
Whole wheat	22.5
Glucose	45.0
Sucrose	16.0
Water	900 cc.

The ingredients are thoroughly mixed and the resultant nutrient sterilized. After cooling to about 35°C. it is inoculated for forty-eight hours or until an acidity of 0.4 per cent is reached. The prepared flavoring composition may be mixed immediately with sweet butter, oleos, and similar products in the ordinary butter working machine until the required texture is secured. This obviates the necessity of using individual jacketed kettles and churning with the particular "starter." His other patented (7) process does away with the necessity for first melting the oils, stearins and neutral lards. The starch base absorbs moisture and in this way the required moisture is worked into globules on the finished product.

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CHAPTER 22

Mobilization of Vegetable Oils

Vegetable fats are ordinarily recovered by pressure after some appropriate preliminary treatment. The plant cell walls must be subjected to high temperature treatment before they are brittle enough to crush under high pressure.

The cell walls are composed of carbohydrates such as sugars and cellulose, and plant protein. The cell walls may be treated with chemical agents to liberate the oil, but one which would attack the cell walls would probably also affect the oil or the residue which is valuable as a cattle food. The other alternative is to utilize a biological method, as enzymes, to attack the cell walls making them water soluble and thus releasing the oil. The process should be simple in principle and operation and should not involve many steps.

Beckman (3) has developed a method for extracting the oil from copra. He makes use of *Bacillus delbrucki* (2) which he describes as a hardy thermophilic anaerobe, obtained from brewers' malt. It attacks sugars, changing them to lactic acid, and producing at the same time enzymes which vigorously attack proteins, converting them to water soluble amino acids. He finds that it works best at 50°C., a condition which of itself prevents the growth of contaminating organisms. The culture is mixed with disintegrated or ground up nuts, a weighed amount of ground limestone, or calcium carbonate, and sufficient water to form a mash, which is then placed in an incubator at the desired temperature with the air excluded. Fermentation takes place and carbon dioxide is given off due to the action of the lactic acid on the calcium carbonate. Oil begins to appear on the surface, the mixture becomes more and more liquid, and after about 6 days gas evolution comes to an end, after which the free oil can be recovered from the residue by filtration. The pH value during the process averages 4.2, which is maintained by use of an excess amount of calcium carbonate. Free fatty acids are produced by lipases formed by the action of molds on the mixture, and they act as catalysts materially increasing the rate of decomposition of the oil. In properly covered incubators, however, the temperature is such that

mold has no chance to grow and the danger of oil decomposition due to lipases is negligible.

Beckman finds that he obtains an oil of high quality by this method, with a normal melting point. The residue, after filtering, is composed of bacteria, calcium lactate and amino acids. The advantages of the biological process over mechanical methods are as follows:

1. The high temperature inhibits the growth of other organisms so that pure cultures are unnecessary.
2. There is no need to watch the acidity due to the excess of calcium carbonate.
3. It is simple and accurate method.
4. A high yield of both oil and cake are obtained.
5. It may be carried on at a low cost.

Lactobacillus delbrucki is described in Bergey's *Manual* (2) as a Gram positive rod, occurring singly or in chains. It forms lactic acid in dextrose, levulose, galactose, maltose, sucrose, and dextrin. It is microaerophilic, and the optimum temperature is given as 30° to 35°C.

In 1921, Alexander patented (4) a process of extracting oil from cocoanuts by a fermentation process at 35°C. and stresses the proteolytic nature of his organism. A large number of the Lactobacilli produce protease as well as the lactic ferment so that any species of this genus would possess this power in varying degree. It would seem that Alexander's method of allowing the mixture to ferment spontaneously and then preserving the proteolytic organisms as a starter culture would produce similar results.

L. delbrucki has been used for the production of lactic acid and in 1913 a patent for the fermentation of dextrose by the action of this organism was granted.

The process for the mobilization of vegetable oil has been patented by Beckman (1) and is fully described and diagrammed in *Industrial and Engineering Chemistry* (3). It is suggested that this process may be of service in mobilizing other vegetable oils now obtained by pressure. In our laboratories we have succeeded in liberating oil from chopped olive meats and from flaxseed meal by this method, but on succeeding trials the organism seemed to lose its ability to perform this separation.

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Section VI

MISCELLANEOUS PROCESSES

CHAPTER 23

Fertilizers, Garbage Fermentation, Beccari System

FERTILIZERS

The bacterial content of garden soil will vary considerable, from 100,000 to 10,000,000,000 bacteria per gram, depending upon the accompanying physical and chemical properties of the respective soil. This is particularly the case when inoculation is performed and new species are added in an attempt to establish themselves in the new environment. Numerous attempts have been made to establish growth in sterilized humus material and several patents have been taken out following this general procedure.

The following genera bring about chemical changes in the soil and are designated directly or indirectly in all of the patent claims.

1. Capable of utilizing carbon, hydrogen, sulphur, nitrogen or their simple chemical compounds.

   Nitrosomonas: Cells oxidize ammonia to form nitrites.

   Nitrosococcus: Cells oxidize ammonia to form nitrites.

   Nitrobacter: Cells oxidize nitrites to nitrates.

   Thiobacillus: Cells oxidize elementary sulphur and sulphur compounds.

2. Organisms capable of fixing free nitrogen of the air.

   Azotobacter: Non-symbiotic bacteria.

   Rhizobium: Symbiotic bacteria on the roots of leguminosae.

Other genera included with the soil group organisms are the Chlamydobacteriales, filamentous, iron containing bacteria, the Chlostridium, Bacillus, Cellumonas, Actinomyces, and Achromobacter.

Fred and Waksman (1) give detailed methods for studying the nitrogen fixing, denitrifying, nitrifying, sulphur reducing, sulphur oxidizing, iron precipitating and cellulose decomposing bacteria in the soil. Agricultural bacteriology is definitely associated with the industrial phases but also constitutes a distinct division in the science of microbiology.

PREPARED FERTILIZERS

Ducommon (4) incorporates the microörganisms present in farm yard manure in a nutrient material consisting of meat extract, peptone and calcium carbonate for the preparation of a commercial fertilizer. Dried vegetable matter, pulverized bone and some fat may also be added. A culture of Azotobacter isolated from the nodules on leguminous plants, is sometimes added followed by incubation periods of forty-eight hours at 30°C. The composition is then treated by exposure to warm air, 35°C., and reduced to a powder.

Another fertilizer composition (5) consists of a neutral mixture of peat, phosphate, protein and carbohydrate materials. The Azotobacter, Nitrosomonas and Nitrobacter are added as "azo" soil taken from niter spots occurring in the soil. *Cellulomonas rossica* and mixed legume cultures are used in preference to pure cultures of the legume organisms. The product should have a bacteria count of about 200,000,000,000 organisms per pound. Such inoculations will support the growth of alfalfa, sweet clover, red clover, black medic, soy beans, cow peas, sweet peas, spring vetch, garden peas, lima beans and alkali clover. There are many similar patents (12).

Makrinoff (6) has prepared a fertilizer using only the Azotobacter for the non-legume crops. Fertilizer can be prepared by digesting humus with phosphate rock and acid phosphate and neutralizing the the mass with calcium carbonate (7).. The moist mass is then inoculated with symbiotic bacteria, Radicola, and the non-symbiotic bacteria, Azotobacter, Nitrobacter, etc.

A process using glauconite in combination with finely decomposed and neutralized 25 per cent dry peat humus, bone meal, malt, sugar, hardwood ashes and flour of sulphur as a nutrient for growing soil organisms was patented in 1924 (8). According to the patent claim the medium is suitable for growth of "peptonizing, ammonifying, nitrifying (nitrite and nitrate), nitrogen fixing, including both symbiotic and the non-symbiotic azotobacters, sulphur bacteria, etc." Cultures for a particular legume may be inoculated with a pure culture of that specific nitrogen fixing bacterium although mixed cultures give the best results. The humus is sterilized by steam or hot air at 125°C. and rendered slightly alkaline with earthy carbonates.

Baldwin and Fred (9) found that the legume nodule bacteria from alfalfa, clover, peas, beans, and dahlia produced an acid fermentation whereas organisms from soy beans and cow peas lowered the hydrogen

ion concentration. A *Bacillus radiobacter* produced a strong acid fermentation of dextrose.

Rudakov states that a decided decrease of the soluble phosphoric acid (10) under aerobic conditions is observed by the inoculation of a nutritive medium, containing a minimum amount of an oxidized mineral compound, with a known amount of soil. This decrease takes place as a result of the reduction of the phosporic acid to phosphorous and hypophosphorous acid, and to phosphine by microorganisms. Isolated pure cultures reduce the acid as above, while the ability varies in the different soils. *Bacillus subtilus*, *Bacillus mycoides*, *Proteus vulgaris* and *Escherichia coli* have been found to be capable of dissolving the phosphates of bone and to a less extent the mineral phosphates (13).

Lipman patented (2) a method for preparing a culture of sulphur-oxidizing bacteria by growing them on a specified culture medium as given:

	per cent
Magnesium chloride	0.01 to 0.1
Dipotassium phosphate	0.05 to 0.5
Ferric chloride	One or two drops of a 10 per cent solution per liter of culture medium
Dextrose	0.05 to 1.5
Sodium nitrate	0.1 to 1.0
Elementary sulphur	1.0

ACID PHOSPHATE

The production of acid phosphate by means of a biological process was suggested in 1914 by Lipman. By providing ideal conditions for the sulphur oxidizing organisms and manipulating the mixtures of sulphur and rock phosphate it was possible to get a mixture where the total P_2O_5 was 15 to 16 per cent, of which 50 to 60 per cent was soluble. The period of incubation was cut down to eighteen weeks. The process is referred to by Joffe (14) as the Lipman process.

NITRATE PRODUCTION

Boullanger (15) studied the production of nitrate by means of biochemical oxidation. He found that nitrification depends upon the concentration of the ammonium salts present.

GARBAGE FERMENTATION

In 1918 a process for the fermentation of garbage after it is neutralized with alkali salts and some organic matter has been added was patented by Wallace and Prescott (11). Fermentation proceeds with the normal microflora although the mass may be inoculated with portions from a previous fermentation. The residuum is dried, inoculated and used as a fertilizer. Dungan patented (3) a process for preparing a fertilizing material from humus, muck, peat, garbage or sewage sludge by inoculation of the material with aerobic bacteria and forcing air through the mass.

In the Beccari system of garbage fermentation the organic refuse is completely aerated and fermentation proceeds with a marked rise in temperature to about 60°C. The volatile products of fermentation are rendered odorless by passage of the gases over earthy substances. The entire fermentation is completed in about forty days and the fermented mass is reduced to a dark brown residue. The fermentation process can be greatly improved by more extensive bacteriological study and the enhancing of the microflora by the addition of suitable salts to buffer and to increase the nutritive value of the garbage liquor.

The Beccari system of fermentation for the disposal of organic waste was established in Florence, Italy, in 1914, with the primary purpose of utilizing farm waste more effectually as a fertilizer. Later the system was successfully applied to the disposal of organic waste produced by municipalities. Mr. Arthur Boniface in 1923, established a Beccari fermentation plant at Scarsdale, New York.

The operation of the American plant was partially successful but operation was suspended pending a visit to the original Beccari plant at Florence. Upon investigation of the character of the organic waste at the Italian plant two important things noted were the small percentage of citrus fruit skins and the almost complete absence of moisture. American garbage contains a large percentage of citrus rinds, a high fat and grease content and a moisture content of about 75 per cent. The acidity of the Florence waste was further reduced by the lime present in the street sweepings.

When samples of American garbage were heated to 65°C., the temperature developed during fermentation, all became definitely acid. By adding sufficient lime to effect neutralization of the ordinary run of garbage, about 3 per cent of a commercial lime,

fermentation began with a rise in temperature to 60°C. within three days and slowly dropped to 54°C. on the thirtieth day. A humus with a 10 per cent moisture content was produced and could be bagged without further treatment. The minimum amount of lime and the maximum depth of layers to which garbage can be piled were determined by further experimentation.

The nitrifying cycle is completed in about forty days taking place in two stages. First the garbage is subject to the action of anaerobic bacteria which decompose and liquefy the mass. The second stage is an aerobic decomposition with the absence of disagreeable odors. A digestion cell is charged ordinarily with about 20,000 pounds of raw garbage in layers approximately 18 inches deep. This amount of garbage requires 300 pounds of commercial hydrated lime and 10 pounds of ammonium sulphate to stimulate the growth of the aerobic microörganisms. The quantity of humus produced is approximately 2400 pounds or 12 per cent of the mass by weight representing a value of $25.00 a ton after grinding.

	per cent
Nitrogen	2.59
Potash	1.76
Phosphoric acid	2.64
Moisture	12.00

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CHAPTER 24

Gas Production, Sewage Treatment, Cellulosic Gas

NATURAL GAS

Natural gas, consisting principally of methane and small amounts of ethane, propane and perchance higher homologues, probably had its origin, along with coal and shale oil, in the slow decomposition of vegetable matter by microörganisms, a process that has been going on for centuries. The fuel value of natural gas ranges around 1000 B.T.U. per cubic foot. The standard heat value for artificial gas is 575 B.T.U. per cubic foot.

SEWAGE GAS

The bacterial count of sewage ranges from 50,000 to 12,000,000 bacteria per cubic centimeter. The complex nitrogenous substances are decomposed into proteoses, peptones, polypeptides, peptides, amino acids, ammonia, carbon dioxide, methane and hydrogen. The colloidal polysaccharides, cellulose and starch, are decomposed to sugars, acids, carbon dioxide, methane and hydrogen. The fat rises to the surface where it is decomposed into fatty acids and glycerin.

Large sedimentaton chambers allow the coarse material to settle out and such material is designated as sludge. The slude is subject to aeration or may be precipitated and dried to prepare a commercial fertilizer. The drying is the expensive part of such a process.

Sewage sludge inoculation is generally used for large scale fermentation to produce a combustible gas. Methane long has been known to exist in sewers, in fact, sewer gas has been used to develop power in internal combustion engines at a decided profit. Charlotte, North Carolina, was the first American city to obtain gas power on a plant operating scale from sewage sludge fermentation.

Briefly, the method consists in passing the sewage through a course bar screen and a clarifier, which acts as a settling tank. The effluent from the clarifier pumped into aerating tanks where it is agitated by air under about 7 pounds pressure. After about four hours the sewage passes into settling tanks. The sludge is removed to the di-

gestion tanks where it remains together with the solids removed from the clarifier, for about two months and is then dried and used as a fertilizer. During the fermentation of the sludge, in the digestion tank, gas is evolved which contains about 70 per cent methane with a heat value of about 600 B.T.U. per cubic foot. The internal combustion engines are standard gasoline units with the carburetors removed and having the gas pipe inserted into the manifold leading to the cylinders.

During one month in the year of operation 88,723,000 gallons of sewage supplied 405,696 cubic feet of gas, producing about 25,000 kilowatt-hours of power. The rate of gas production was too low to operate the engine continuously so motor driven units were run until the gas holders were filled.

TABLE 15

POPOFF'S ANALYSIS OF SEWAGE GASES

	TWO WEEKS	SEVERAL WEEKS
Carbon dioxide	25.70	34.07
Methane	14.42	37.12
Hydrogen	14.36	1.06
Nitrogen	45.52	27.75

GAS FROM CELLULOSIC FERMENTATIONS

The fermentation of cellulose was first attributed to microörganisms by Mitscherlich (1) in 1850, but the connection between cellulose fermentation and methane production was not pointed out until 1875, by Popoff (2). He inoculated Swedish filter paper with sewage sludge and collected the evolved gases which he analysed (see table 15).

In 1904 Omelianski (3) published his methods for the separation of the "hydrogen bacillus" from the "methane bacillus." It is not unlikely that the gaseous products of cellulosic fermentation are due to secondary fermentations by other organisms.

The production of combustible gases as the result of controlled microbial action on vegetable tissue is indeed a problem for the engineer. In 1918, Langwell patented (4) a process for the manufacture of combustible gases by the fermentation of straw and sulphite pulp by microörganisms.

The simplest formula for production of methane and carbon dioxide from carbohydrates is:

$$C_6H_{10}O_5 + H_2O = 3CO_2 + 3CH_4$$

calling for a 1:1, carbon dioxide to methane ratio.

Later, in 1920 Fowler and Joshi (5) experienced vigorous cellulose fermentation with gas production in materials containing hemicelluloses. Maximum gas production occurred at 30°C. with a daily evolution of combustible gas equal to 80 per cent of the volume occu-

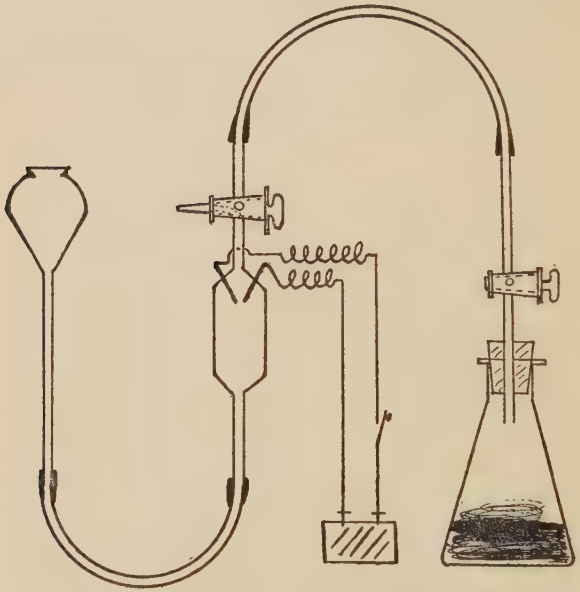

Fig. 7. Small Explosion Pipette

pied by the fermenting material. Of this gas 80 per cent was methane and the average calorific value calculated from the composition of the gas was 1.45 times that of coal gas (see table 16).

All of their work was carried on with cellulose fermenters, not fully described, but the "bacterial emulsion" was prepared by inoculating banana stems and skins with sludge from a septic tank. It required four days for the fermentation to begin. An acidity of more than 1 per cent had a retarding effect on future fermentation. The banana skins gave the largest yield of combustible gas and the hemicelluloses,

generally, are claimed to have given the most satisfactory fermentations. Only acetic acid was detected in the fermentation. The type organism was likely a thermophilic anaerobe.

Langwell and Hind (6) describe a cellulose fermenter, facultative anaerobe, producing 18,000 cubic feet of gas per ton of cellulose fermented. The organism was a slender rod 0.4 by 4.0μ, fermenting xylose as well as cellulose with an optimum temperature at about 60°C. The culture became progressively pure at this temperature. Mazé (7) obtained combustible gases by inoculating potassium acetate and sodium butyrate, respectively, with a culture of bacteria isolated from decaying leaves.

It has been shown (8) that calcium acetate solutions inoculated with sewage sludge produces a gas of 2 parts methane to 1 part carbon dioxide.

TABLE 16

Gas Production in Materials Containing Hemicelluloses

	METHANE	HYDROGEN	CARBON DIOXIDE
Filter paper	81.9	14.5	3.5
Newspaper	88.8	4.4	6.8
Letter paper	78.6	12.6	8.7
Banana skin	80.6	3.1	7.4
Banana stems	87.0	5.4	7.5

The pH range of gaseous fermentation is best at a value between 3 to 10 usually in the absence of organic nitrogen, at about 35°C.

Boruff and Buswell (9) prepared four digestion mixtures, using cornstalks which had been shredded, soaked, boiled, and boiled in lime, respectively. The stalks were decomposed to the extent of 35 to 50 per cent and an equivalent weight of gas was produced. The best results were obtained with material which had been soaked in lime water. Gas samples contained from 50 to 70 per cent methane. Such a gas has a calculated heat value of more than 500 B.T.U. per cubic foot, equal to that of coal gas. Sewage inocuations were again used as the source of the microörganisms. The process was carried out anaerobically, hence the type organism, again, a clostridium. One ton of cornstalks has yielded from 10,000 to 20,000 cubic feet of gas.

Heukelekian (10) obtained digestion of approximately 80 per cent

of cellulosic materials in three weeks' time, while nitrogenous material requited several months for digestion. The carbon dioxide and organic acids produced during the first period of fermentation at 20°C.,

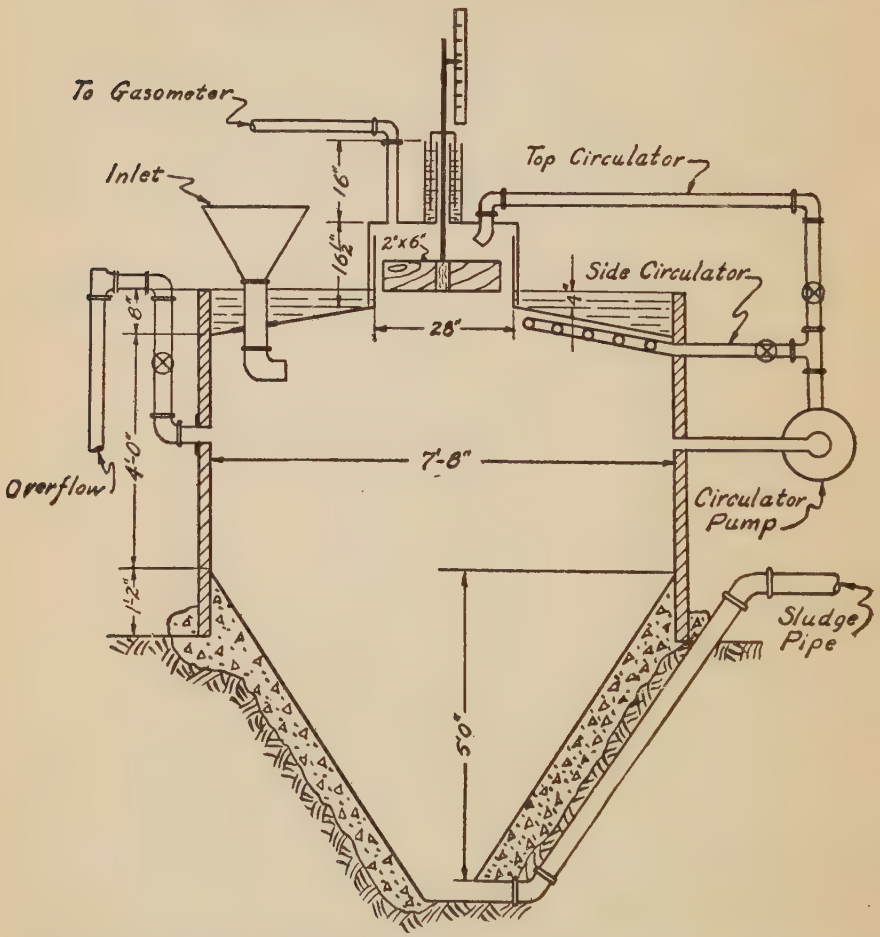

Fig. 8. Cross Section of Experimental Tank Showing Arrangement of Circulators to Prevent Formation of Scum Which Retards Gas Production

(Courtesy of Dr. Buswell and Society of Western Engineers)

frequently lowered the pH value from 7.0 to 5.5, in the second period the pH value rises again to 6.5 or 7.0 with an increase in methane

production and in the third period the more complex material undergoes microbial decomposition, at a pH value of about 7.0 to 7.5 with a definite increase in gas volume and the percentage of methane. The clostridia, anaerobic spore formers, are indicated as the type organisms.

Industrial wastes containing phenol, 25 p.p.m., are detrimental to gas production. Other chemicals will affect the fermentation according to the concentration.

	p.p.m.
Chlorine	50–5000
Magnesium sulphate	360
Sodium chloride	5,500–30,000

TABLE 17

PERCENTAGES OF GASES IN SOLID SEWAGE

TEMPERATURE	TIME IN DAYS	PER CENT CO_2	PER CENT CH_4
45°C.	7	—	—
	8	26.2	65.0
	11	20.6	73.2
	20	17.3	76.5
50°C.	8	—	—
	11	24.7	66.7
	20	14.1	81.0
	7	27.2	61.4
55°C.	8	23.4	65.0
	11	19.7	64.7
	20	16.3	82.5

Total digestion time eighteen to twenty days with 50 per cent reduction of volatile matter.

Anaerobic gas metabolism results in the production of carbon dioxide, hydrogen, methane, hydrogen sulphide and nitrogen. Other gases produced by microörganisms are ammonia and phosphine.

Heukelekian and Rudolfs (11) in 1930 used sewage solid, seeded and unseeded, for digestion at thermophilic temperatures in order to obtain information as to the rate of digestion as well as the quantity and composition of the gas evolved. Digestion of fresh sewage solids at temperatures of 45° to 55°C. was readily demonstrated. The time required for digestion was materially shortened when the inoculant is

prepared under thermophilic conditions. Solids seeded with ordinary mesophilic inoculants do not digest rapidly in the thermophilic range, but thermophilic inoculants produce higher yields of a 70 per cent combustible gas. The percentage of gases, shown in table 17, produced at various temperatures, was rearranged from the above investigators' report.

The pectocelluloses (flax, hemp, ramie, sisal), the lignocelluloses (jute and straws) as well as cuto and adipo celluloses (skins of fruits) undergo gaseous fermentations. In addition to the production of power gas from cellulose fermentation, other products are industrial alcohol, organic acids and synthetic manures.

The use of sewage gas for heating purposes has been undertaken by the city of New Castle, Pennsylvania. The Imhoff tanks treat 4,300,000 gallons of sewage with a production of from 3400 to 36,000 cubic feet of gas daily. The city has a population of about 60,000.

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CHAPTER 25

Commercial Preparations

CHICKEN FEED

A bacterial preparation consisting of cellulose fermenting bacteria isolated from the gizzards of chickens was patented (1), in 1926, by Reinhold and Fultz. The method described in the patent for isolation of the cellulose fermenters would result in the growth of many other organisms unable to attack cellulose. The growth is washed off of plain agar plates with distilled water and the suspension dried at 30°C. Rock phosphate and cornstarch are added to give bulk and the dry mixture is added to chicken or other livestock feed.

VERMICIDE

A preparation for infecting vermin with cultures of *Bacillus danysz* grown in a meat extract broth was patented by Palma in 1916 (2). The broth is inoculated and incubated for forty-eight hours at 35°C. Immediately before use a small amount of nitrate is added to the culture.

The organism belongs to the genus Salmonella and is a motile rod. Other preparations of this type use an organism, pathogenic for mice, known as *Salmonella typhimurium* but since Palma's organism does not reduce nitrates it is more likely that his organism should be designated as *Salmonella enteritidis*. There is no mention in the patent as to where the original culture was obtained. The authors note successful results with the use of such cultures of living bacteria but the relation of epizootics in rodents to the public health of the community question the advisability of their general use.

COMMERCIAL STARTERS

Commercial starters may be prepared in the liquid or dry form. The dry preparations are made by mixing a liquid culture or suspension with some inert material, usually a carbohydrate. This mixture is dried at a low temperature.

The liquid preparations are more generally used because they

allow the organisms to grow and multiply and usually produce a more active fermentation. Liquid cultures that are allowed to stand over a long period of time lose their vitality due to the products of cell metabolism which are injurious to the cells.

Reserved portions of the major fermentation are possibly the best starter cultures, where obtainable.

YEASTS

A patent (3) to prevent undue clumping in the propagating baker's yeast claims the addition of ammonium sulphate, 1 per cent of molasses, neutralized with lime to form calcium sulphate. The preparation is buffered with phosphates. Another patent (4) uses lactic acid forming bacteria to clarify molasses and increase its assimilable nitrogen for use in yeast manufacture. The organic acid formed is neutralized with lime and ammonium sulphate is added to precipitate the calcium which is separated from the molasses. Whitney (7) has patented a process for preparing dry yeast.

All the yeasts of any economic importance are included in the genus Saccharomyces. The most common commercial species is the *S. cerevisiae*. There is a marked variation in the shape of the yeast cells and some workers have attempted to separate and classify the cells upon this basis. In industrial fermentation it is common to refer to type cultures of certain strains that are known to have a marked physiological activity. The fermentation of the various carbohydrates is perchance the most satisfactory manner for the tentative separation of the numerous groups.

LACTOBACILLI

Earp-Thomas has patented (5) the preparation of a fluid oil product containing Lactobacilli together with an emulsifying agent and some nutrient as lactose or dextrose. The use of *Lactobacillus acidolphilus* and *Lactobacillus bulgaricus* is discussed in Chapter 27. There are numerous patents pertaining to the use of these lactic organisms, usually as medicinal remedies to overcome intestinal putrefaction. In 1927, James (6) examined 107 samples of *L. acidophilis* and *L. bulgaricus* preparations on the open market. Only 13 produced species claimed on the label in reasonably pure form, 15 preparations had sufficient organisms to regard as predominating culture, whereas the remaining were not representative of the species claimed.

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Section VII
MICROBIAL THERMOGENESIS

CHAPTER 26

Microbial Thermogenesis

INTRODUCTION

Since all microörganisms secure their energy for normal metabolism by a direct chemical oxidation of chemical compounds or through intramolecular rearrangement as is encountered particularly under anaerobic conditions, it becomes evident that a small amount of heat production occurs in all stages of microbial decomposition. The accumulation of heat to a point at which it becomes appreciable is a direct function of the mass of organic material and its insulating coefficient. It must not be conceived that the heat is due to the bacteria, per se, for the bacteria are regarded as being poikilothermic, but rather that the energy of the microbial metabolism is, in a large part, determined by the respective substratum. Heat production is due to the lack of chlorophyl in the microbial cell and the inability of most microörganisms to store the energy of light in the form of organic substances.

Heat production in moist organic materials has been definitely associated with microörganisms for the past half century. The temperatures in these fermentations seldom exceed 60°C. Investigators have demonstrated the ability of microörganisms to produce such temperatures, although some workers have attributed no part in the heating process to them. Heating by bacteria up to a maximum of 80°C. is generally accepted although it is likely that a simple chemical oxidation occurs in addition to the microbial activity.

Browne in a recent bulletin (1) gives a complete review of the chemical, enzymatic and microbial theories of "spontaneous" combustion in relation to agricultural products. The present attitude toward "spontaneous" heating and possible ignition may be designated as the chemical-microbial concept, namely, that bacteria growing and multiplying on the organic materials produce secondary products of metabolism which may be responsible for the actual ignition under given conditions.

A definite rise in temperature has been observed in paper pulp, box

board, bark, fish scrap, tankage, artificial fertilizers and manures, stall manure, fish guano, hay, clover, alfalfa, grass, tobacco leaves, malt, ensilage, bagasse, cereal grains (oats, corn and wheat), bran, hops, tobacco, seeds, concentrated cattle foods, molasses, skins, hides, tankage, hair, wool, cotton, flax, hemp, cacao, straw, shoddy, jute, kapok, sisal, etc.

THERMOGENESIS

Heating may or may not be desirable. Whereas the normal thermogenic fermentation of silage, tobacco, cocoa and coffee beans is

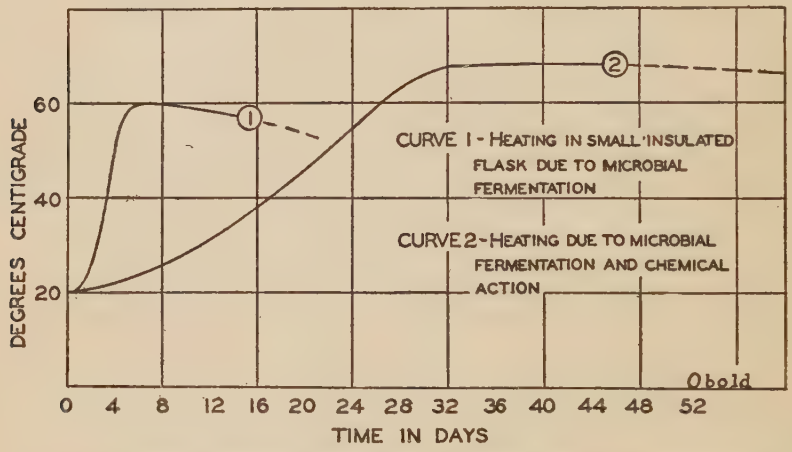
DEGREES CENTIGRADE
60
40
20
0 4 8 12 16 20 24 28 32 36 40 44 48 52
TIME IN DAYS
1
2
CURVE 1 - HEATING IN SMALL INSULATED FLASK DUE TO MICROBIAL FERMENTATION
CURVE 2 - HEATING DUE TO MICROBIAL FERMENTATION AND CHEMICAL ACTION
Obold

FIG. 9. CURVES SHOWING THE RATE OF RISE DURING THERMOGENIC FERMENTATION

beneficial, the loss from heating in stored hay, grain and textile fibers is as important an item as the loss from actual ignition (2).

While the carbohydrates are usually associated with thermogenesis, James (3) demonstrated that a certain amount of nitrogenous metabolism is demonstrable. About 30 per cent moisture is most favorable for the heating of agriculture products. The heating does not take place in the absence of air or oxygen. The same investigator describes an apparatus suitable for the study of microbial thermogenesis in which temperatures of 60°C. have been repeatedly obtained by using corn meal inoculated with various strains of typical air and soil type organisms such as *Bacillus subtilis*. Tobacco fermentation with

about 25 per cent moisture will give a temperature of from 48° to 70°C. in about three days. Cacao and coffee beans during fermentation reach about 45° to 50°C. in six days.

Hill (4) found that in twenty-four hours bacteria produced approximately 1.3 calories of heat per gram of milk during souring. Shearer (5), working with *Escherichia coli*, found that when amino acids were

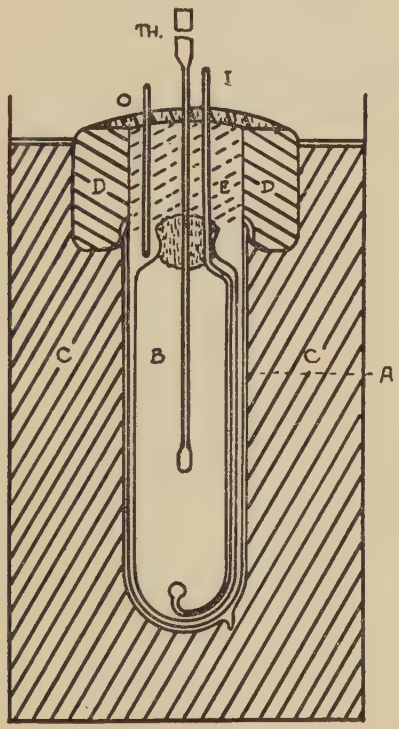

FIG. 10. CHAMBER FOR THE STUDY OF THERMOGENESIS
(After James)

available there was no heat produced but when casein was attacked an appreciable amount of heat was liberated. According to these results microbial thermogenesis is an indication of less economical growth than that attained in the normal metabolism of the respective organisms. A majority of these organisms are facultative anaerobes which when placed in an environment exclusive of oxygen may begin

to grow anaerobically with greater heat production. It is noted that Shearer obtained the greatest amount of heat in a peptone glucose chalk medium. Langwell and Hind (6) were unable to get heating during a cellulose fermentation unless ammonium sulphate and calcium carbonate were present. The demand for oxygen was so great that the SO_4 was reduced to H_2S.

MICROÖRGANISMS

The organisms responsible for the heating of moist organic materials are widely distributed in nature. It is quite possible that all organic

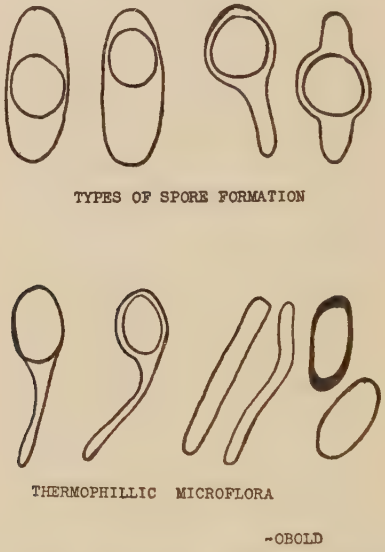

Fig. 11. Typical Spore Formation with Thermophilic Microflora

materials with a microflora undergo slight thermogenic fermentation in the presence of sufficient moisture and when stored in large quantities for long periods of time. Any organism or group of organisms that can attack the given substratum will produce appreciable amounts of heat during the period of attack.

We include three genera as capable of producing a thermogenic fermentation although there are numerous other species of bacteria and molds (Aspergilli) capable of producing the same effect:

1. Bacilli, aerobic, spore forming rods, facultative thermophiles.
2. Clostridia, anaerobic, spore forming rods, producing many spheroid and hyaline bodies with long slender rods, facultative thermophiles.
3. Escherichia, small, Gram negative, rods, facultative anaerobes.

In a typical thermogenic fermentation when the temperature rises to about 60°C., the predominating organisms are slender rods 3 to 20 microns long (averaging about 6 microns) by 0.6 to 1.0 microns in width, and with oval terminal spores. The microscopic field shows many hyaline bodies separated from the vegetative portion which increase in number as the temperature rises. Many thread forms are observed about 50°C. The cells are Gram positive at 40°C. but stain indifferently as the temperature rises.

THERMOGENIC FERMENTATIONS

Following the floods in New England in 1928, investigators (7) observed temperatures of 74°C. in hay mows in the flooded region that had undergone microbial fermentation.

One case (8) of heating with ignition has been observed in a manure pile. The interior of the pile averaged only 51°C. but following the introduction of pure oxygen into the mass a definite rise to 190°C. occurred. Further aeration resulted in cooling of the mass. The charred straw when exposed to air burst into flame.

SUGAR

Refined sugar has been charged with heating but the evidence is not conclusive. Caramelization and melting, which might be expected in a thermogenic fermentation, have not been observed in large sugar storage although high bacterial counts are not infrequently found. The presence of sugar in jute bags appears to have no relation to any increase in the "spontaneous" ignition fire hazard.

BAGASSE

Fresh bagasse, in 1000 cubic foot bins and with about 48 per cent moisture and about 4 per cent polarization, gave a reading of 43°C. in twenty-four hours and 48°C. in seventy-two hours, reaching a maximum of 60°C. at which temperature it remained constant for about six days, then gradually began dropping to ordinary temperature (9). Thom and Lathrop (26) found Psilocybe on bagasse.

MINERAL, VEGETABLE AND ANIMAL OILS

Mineral oils are not subject to bacterial fermentation although mold has been isolated in several instances. No heating was observed.

Vegetable oils may be hazardous due to the simple chemical oxidation of the unsaturated fatty acids in air or oxygen. The hazard from a bacteriological viewpoint is the possible fermentation of the by-products as linters, hulls, presscake, tankage, etc. All seeds heat if moisture is present. Cotton seed which has been crushed will ferment and heat rapidly, exposing the oil to the air for oxidation and possible ignition. Corn oil is a by-product of the starch, glucose and alcohol industries. Contrary to some opinions we have not found this oil subject to bacterial fermentation. Peanut hulls are a valuable cattle food and must be handled carefully to avoid overheating and ignition. Cocoanut press cakes are known to heat and copra ferments due to the proteolytic microflora. Garbage oils have a definite nitrogen content and undergo rapid fermentation when the moisture content is above 8 per cent.

Oils from marine animals (herring, salmon, sardine, cod, whale, seal) are subject to putrefactive changes due to the nitrogen present. Fish scrap is used extensively as a commercial fertilizer. Such scrap readily undergoes extensive heating, particularly when the moisture content exceeds about 13 per cent (25). Acidulated scrap is less subject to spontaneous heating although it may contain as much as 50 per cent moisture.

HEATING OF HOPS

The heating of hops during proteolytic decomposition has been attributed to the production of trimethylamine (10). Traces of trimethylamine have been detected in cultures of a *Pseudomonas putida*. The low boiling temperature of about 3.5°C., and the inflammable vapors of this amine raise a question as to its relative fire hazard in heating materials during proteolytic decomposition. Trimethylamine has a pentrating, fishlike odor and can readily be detected in old cultures of *Pseudomonas aeruginosa*. It is produced in the decomposition of the nitrogenous residue, vinasse, from waste beet sugar.

FEEDS

Dairy feeds are known to heat and the moisture content is a large factor in preventing this heating. Alfalfa meal with corn, oats and molasses is more subject to heating than alfalfa meal and molasses alone. Such a preparation may contain as much as 40 per cent moisture and is therefore subject to fermentation processes. If the moisture is below 12 per cent the heating is greatly retarded. There should be a limit of allowable moisture agreed to by the respective feed manufactures. Such feeds are increasing in popularity and more concerns will enter this field by developing by-products of food manufacture containing suitable nutrients.

WOOL

Heating with charring and the detection of a glow has been observed in contaminated wool while in storage. It is impossible to state that the charring was definitely the result of microbial heating although heating with a definite odor of putrefaction has been observed in a 3000-pound mass of wool (11). Saturation is attained when the moisture content reaches about 33 per cent. Dry wool immersed in water yields 24.1 calories of heat per gram (12), that is, 1 pound of dry wool when completely wetted is sufficient to raise the temperature of 1 pound of water as much as 24°C. or 43°F. Under normal conditions, however, absorption takes place slowly and the heat effect would be less noticeable.

B. subtilis and *B. mesentericus* are found upon cultural examination of the hairs, as are also the Actinomycetes and molds of the genera Penicillium and Aspergillus. Woolen cloth that has been subject to mildew frequently fails to take the dye or conversely may become too deeply dyed. This indicates a chemical change in the composition of the hair as a result of microbial growth.

SKINS

A report (13) of spontaneous heating in 2400 sheep skins stored for eleven days in a Boston wharehouse states that no actual ignition occurred although smoking of the hides was observed. The hides had been rough tanned just before shipment from Australia. The oil content of a sample was 12 per cent as compared with a normal of about 8 per cent. The hides had been retanned with logwood and iron and dyed with nigrosine. The time that elapsed before heating

was observed is a difficult factor to explain on a straight oxidation hypothesis, but any explanation is lacking in finality.

SAWDUST

Sawdust when green and sappy or damp is subject to microbial attack as evidenced by the discoloration. The major part of such a decomposition is brought about by the higher fungi (24). There are references to the production of phosphine gas to account for spontaneous ignition of sawdust and in particular, ice house fires. There is no good evidence that such a gas has been detected although its production is by no means precluded. Production of phosphuretted hydrogen, however, would serve as a connecting link between bacterial decomposition with attendant heating and spontaneous ignition of the mass.

HEMP, JUTE, SISAL

Hemp, jute, sisal and other complex cellulosic fibers will heat if the moisture content is raised to about 40 per cent. Such heating reaches a maximum of approximately 60°C. There is no instance of heating of this kind that reached the point of ignition or even charring, although the heating may cause a total loss. Sisal hemp heats quite rapidly in the presence of sufficient moisture. Jute, a lignocellulose, heats much more slowly. In practice it is assumed that three days after hemp is wet it has become heated and damaged to such a degree that it will have little salvage value. Jute is readily salvaged by drying, although a 400-pound bale of jute will take up more than its weight of water. These fibers are not readily attacked by the anaerobic cellulose fermenters because of the complex structure of the fiber cellulose. All of these fibers are prepared by water retting in which the pectic substances have been fermented leaving only the hemicelluloses and the lignocellulose complex for further bacterial fermentation.

HAY

While it is generally agreed that hay is subject to spontaneous ignition, the relation between the microbial heating and the actual ignition has been a debateable question. Moist hay serves as nutrient for extensive microbial growth permitting fermentation with the attendant thermogenesis. How the temperature of the haystack rises above the thermal death point (80°C.) of the microflora is unknown.

Stuart and James (14) studied the effect of salting on the microbial heating of alfalfa hay. Laboratory experiments were conducted with moistened, salted, and unsalted hay. No heating was observed under anaerobic conditions. Under aerobic conditions 30 per cent moisture was found to be the optimum for heat production. The addition of 5 per cent salt failed to prevent microbial heating, although salting was found to induce a considerable delay before heating commenced. The addition of 2 per cent salt prevented bacterial growth but failed to prevent mold development although the growth of the mold was also considerably delayed. Their results indicate that the prevention of molding and heating of hay by the addition of salt would only be efficient under such conditions as would allow drying below the critical point for mold developed within the short period of delay caused by the added salt. The addition of sufficient salt would prevent the use of the hay as a cattle food.

TOBACCO FERMENTATION

The texture and aroma of tobacco are definitely affected during the curing process. The attendant heating or sweating of the leaves should not exceed 50°C. although temperatures in this period up to 60°C. are observed. Carbon dioxide and ammonia are produced. The tobacco may be moistened with sugars, syrups, malt extract, honey or alcohol, resulting in the formation of aromatic esters and thereby improving the quality with special reference to flavors. Microörganisms associated with tobacco fermentation are: *Bacillus subtilis*, *Proteus vulgaris*, *Bacillus mycoides*, *Pseudomonas fluorescens*, *Bacillus polymyxa*, *Lactobacilli*, *Torula acetoaethylicus*, *Cladosporium*, *Aspergillus fumigatus*, *Aspergillus candidus* and other fungi. Bedortha used a tobacco extract culture of microörganisms isolated from fermenting tobacco of a superior quality to give to the inoculated tobacco leaf a more desirable flavor than it would posess if allowed to ferment naturally. His patent (15) in 1918, describes the preparation of a tobacco extract medium made by boiling from 0.5 to 1.5 pounds of tobacco leaves in 50 pounds of water. The tobacco extract is sterilized and then inoculated with the desirable microörganisms. Aerobic organisms are used although no description of the desired organisms is given. The time required will vary with the different cultures but is usually about two or three days or until an appreciable amount of free ammonia is detected. Culture medium equal in weight

to about 80 per cent of the dry weight of the tobacco is sufficient. Erslev (16) in 1920, patented a process for improving tobacco which comprises treating tobacco with a carbohydrate containing culture of lactic acid or butyric acid microörganisms. Suitable culture media for growing the desirable microörganisms are malt extract, milk, whey and sugar broths. The microörganisms may be isolated from milk, soil, hay, or leaves by ordinary plating methods. Type organisms included in the patent are: *Proteus vulgaris, Pseudomonas fluorescens, Bacillus polymyxa, Torula acetoaethylicus* and *Cladosporium.* The dried leaves are infused with fresh cultures of the above organisms or sprayed and then dried to a degree of moisture suitable for fermentation.

Waksman (17) believes the fermentation is probably not microbial but enzymatic in character because at 63°C. the tobacco becomes sterile athough fermentation goes on. While such a temperature is high for a desirable fermentation of tobacco leaves we have a culture of *Lactobacilli* fermenting very actively at 60°C. It is noted that the decomposition of tobacco leaves in the presence of chloroform gives an acid reaction while a normal fermentation by microörganisms results in a basic reaction and the formation of pyridine and amino bases from the nicotine. An alkaline reaction does not take place in the absence of sufficient moisture. Cigarettes consist exclusively of fermented tobacco. Since both leaf enzymes and microörganisms take part in the fermentation it is impossible to say which is the most important.

Thom (18) reports an *Aspergillus niger* capable of reducing tobacco leaves to a powder. Sartory (19) found *Aspergillus fumigatus* on moldy cigars. True (20) found *Aspergillus candidus, Aspergillus subgriseus* and *Stemphylium castanea* on cigars and attributes their presence to the paste.

COFFEE BEAN FERMENTATION

Robinson patented (21) a process for treating coffee berries to improve the flavor and palatability of the ultimate beverage. A suitable culture for inoculation is prepared from a carefully selected coffee by placing a few berries in sterile water and transferring to nutrient agar plates which are incubated and then sub-cultured. Fermentation is facilitated by spraying the coffee berries with warm water in order to swell and soften them and also to initiate the mold

growth. Type organisms usually present are *Aspergillus ochraceus*, *Aspergillus niger*, *Aspergillus tarnarii* and *Aspergillus flavus* according to Thom (18).

COCOA BEAN FERMENTATION

Cocoa beans undergo a thermogenic fermentation when mixed with plantain leaves and sufficient moisture is present. According to Loew (23) the process of fermentation depends upon an alcoholic fermentation by yeast cells growing on the syrup from the cocoa beans and the subsequent oxidation of the alcohol to acetic acid by the acetobacter. The tannin is oxidized which accounts for the brown color of the bean. The temperature may rise to 55°C. in about six days.

TANBARK FERMENTATION

The manufacture of lead carbonate by the old Dutch process requires the use of spent tanbark in large quantities. The method consists in placing lead buckles in earthen pots partially filled with about 2 per cent acetic acid. The pots are covered with lids and placed in large trays which are in turn placed in a pit, covered with tanbark and built up to a height of 80 feet in alternate tiers of pots and tanbark. The process lasts approximately 110 days and seldom exceeds four months with a 60 to 80 per cent efficiency in the corrosion. The fermentation starts at room temperature and rises to 70°C. in about thirty days. This is followed by a decrease in temperature to 55°C. at which time the stack is drawn. The high temperature causes the acetic acid to volatilize and form lead acetate and the carbon dioxide produced by microbial metabolism combines with the lead acetate to form lead carbonate.

The pH value of the tanbark in an active stack is about 3.5. The moisture content of the tanbark in an active stack is about 53 per cent, moist enough to mold in the palm of the hand but no water can be expressed even with some pressure. If the tanbark is too wet a butyric acid fermentation develops in about ten days at 20°C. The junior author has demonstrated that aerobic, anaerobic and microaerophilic growth is easily obtained with abundant growth in all cultures up to about 75°C. The predominating organisms are rods, 3 to 8 microns long by 2 microns wide. As in practically all of the thermophilic fermentations, many long thread forms, about 18

microns, are observed as well as many hyaline oval bodies which increase in number with the age of the culture. These organisms are classified as thermophiles due to the temperatures at which they grow best, namely, about 65°C. They go into their spore state and remain dormant at about 75°C., but are readily subcultured at lower temperatures. On culture media they resemble typical air-soil type organisms, and are strongly proteolytic and reduce nitrates to nitrites. The same tanbark may be used over again with entirely satisfactory results in the corrosion of the lead, indicating that microbial nutrients are still available. It is customary to mix the old and new tanbark in all stacks. Humidity plays a part in the corrosion and the grade of acetic acid is extremely important. The odor of butyric acid can be detected in waterlogged tanbark and such a stack is said to have "gone bad." Occasionally two stacks that are built up out of the same tanbark will give very different yields even though they may be built by the same workmen and subject to the same conditions of pressure, humidity and temperature.

The high temperature organisms of fermenting tanbark are not actively thermogenic but the tanbark is an excellent insulation for the heat of chemical reaction. The carbon dioxide production by the microörganism is of paramount importance and according to our experiences the only part that the microörganisms have in this particular process. According to Grieg-Smith (22) the presence of nitrogenous matter regulates the evolution of carbon dioxide in the stack.

SPONTANEOUS IGNITION

Some organic materials heat and ignite under certain conditions without the aid of any external source of heat. If the heat is not dissipated the temperature rises, oxidation is accelerated and ignition may follow. The classical demonstration is the spontaneous ignition of linseed oil soaked cotton waste to which a small amount of a chemical dryer has been added. The temperature rises slowly to about 180°C., after which the rise becomes more rapid and is followed by smoking, glowing and, with a gentle breeze it bursts into flames.

The Mackey apparatus is generally used to determine whether a material is subject to spontaneous combustion by purely oxidative processes. It consists of an annular boiler containing the bath liquid, usually water at 100°C., in the interior opening of which is a wire gauze cylinder for holding the material to be tested and also tubes for

supplying air to the sample. Spontaneous heating is indicated by a rise in the temperature of the test sample above that of the container. A somewhat more satisfactory procedure consists in slowly heating the material to be tested within a chamber whose temperature is maintained a little below that of the sample after it begins to heat. This provides insulation against heat loss, comparable in part to what is found in a much larger scale, as in warehouse storage. The Mackey apparatus determines only the susceptibility of the material to spontaneous heating at a given temperature but is not applicable to a study of microbial thermogenesis.

Ignition as a result of microbial processes has not been demonstrated although microbial heating does occur. The true intracellular enzymes (zymases) produce heat during fermentation although the temperature seldom rises above 65°C. At such a temperature the ignition of the substrate is not likely to occur. The spontaneous ignition of an organic substrate as the direct result of any fermentation process appears to us to be highly improbable.

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Section VIII

MICROBIAL FOOD PREPARATIONS

CHAPTER 27

Vitamin Foods, Milk Products, Acid Beverages, Acid Curd Cheese, Ripening of Cheese, and Bread

VITAMIN FOODS

The production of synthetic food with a definite vitamin content can only be furthered by biological methods, that is, by the use of bacteria, yeasts or molds. It is possible to produce a protein-like body as well as an increase in the fat content by incubation of suitable organisms on a carbohydrate substrate. Any mycologist or physiological chemist can appreciate the commercial possibilities of vitamin-containing foods and other desirable features of a biological product. Ordinary yeast, *Saccharomycetes cervisiae* and the yeast powder of Harris are outstanding sources of vitamin B and serve as an excellent source also of the "P-P factor" (1). The former vitamin, however, is the one most widely distributed in nature and consequently the most unlikely accessory food substance to be lacking in the ordinary diet. The assimilable proteins of the microörganism together with the diversified mineral content are also to be reckoned with in the diet.

MILK PRODUCTS

The production of lactic acid is one of the most common fermentations occurring in nature. Milk readily undergoes lactic fermentation with a reduction in the sugar content and the precipitation of the casein, usually referred to as curding. The bacteria bringing about this change belong to one of two recognized genera, the Streptococcus and the Lactobacillus. Other species and genera are present but are usually outgrown by the lactic acid organisms and consequently rarely develop. Putrefaction is prevented by the strongly acid reaction produced.

The optimum temperature range of the lactic acid streptococci is between 20° and 40°C., whereas the lactobacilli usually find an optimum between 40° and 60°C. Of recent years *Lactobacillus bulgaricus* and more particularly *Lactobacillus acidophilus* have been used in

whey cultures to combat putrefactive changes in the intestinal tract. The lactic ferments are widely distributed in nature and occur in sauerkraut and numerous other food fermentations.

A method of coagulating or curdling milk by means of enzymes produced by the growth of the fungus *Mucor rouxii* upon a suitable culture medium such as rice was patented (2) in 1927. The rice is prepared by cooking in three times its weight of water or water and milk mixture until the liquid has been absorbed by the rice kernels. Fresh milk or milk powder may be used. After the mass has cooled it is inoculated with the mold spores and allowed to incubate under suitable conditions. If the final product is to be made by grinding the mold growth together with the rice on which it is grown, it is preferable to spread out the inoculated medium in 1 or 2-inch layers on shallow pans having a large surface area. The pans are covered to restrict free access of air and to delay the formation of spores which due to their dark color, would tend to impart an undersirable tint to the ultimate product. Under proper conditions of temperature and aeration the mold mycelium permeates the rice medium in about two days' incubation. The resulting cake consisting of mold mycelia and rice medium is broken and ground into a fine powder, preferably of 200 mesh to facilitate the action of the enzyme. The powdered product is added to milk in varying quantities depending upon the activity of the elaborated enzyme. *Mucor rouxii* usually produces a branched sporangiophore and rarely produces spores at room temperatures. Under anaerobic conditions it is capable of producing small quantities of alcohol.

Groll patented (3) a process for preserving the lactobacilli and other microörganisms by incorporating them in a sugar preparation. The method consists in boiling sucrose to 40° or 50° of the sugar scale, with continual washing to prevent crystallization, then pouring the melted sugar, to which some potato starch or glucose may be added, onto a marble slab to cool. The fondant retains about 20 per cent moisture for varying periods of time. The culture of the desired organism is grown in a semisolid state and mixed with equal parts of the fondant. Such preparations have produced normal Yoghurt milk in eleven hours at 42°C. after two years' preservation.

ACID BEVERAGES

Lactic acid is essentially a product of bacterial fermentation and its production affords protection against proteolytic decomposition by

the common air-soil type microörganisms. Acetic acid is frequently produced in small amounts during a lactic fermentation.

The *Lactobacillus bulgaricus* is used in the preparation of numerous food products made from milk. Seaver patented (4) a food composition made by combining *Lactobacillus bulgaricus* with finely ground grain or fruit residue, milk, protein and bran.

Dr. Wiley patented (5) a process of making an acid food of pleasant flavor and nutritive value by artificially souring skimmed milk with *Lactobacillus bulgaricus*, homogenizing the fermented material and quickly drying to give a dry powdered product.

Grelck patented (6) a method of making a malted-acid-milk product. The casein of the soured milk is precipitated and malt extract is added. The product is evaporated at a temperature that does not destroy the malt diastase.

Wahl patented (7) a process of preparing food products by mixing ungerminated vegetable products, insoluble phosphates, proteins and carbohydrates with water and incubating at about 50°C. to enhance the growth of Lactobacilli. He also patented (8) a process of moistening with acidulated water cereal matter (bran) and incubating the mass between 45° and 60°C. The dry product represents a sugar-lactic acid-extract of bran produced by the action of the diastase on the starch and a lactic acid fermentation producing acid phosphates. The same author describes (9) the preparation of a maltless non-alcoholic beverage consisting of ungerminated vegetable matter, lactobacilli, starch, vegetable phosphates and proteins. The lactic liquor is prepared by subjecting bran mash to the action of *Lactobacillus delbrucki*. The following materials are used in the proportion indicated.

Bran	500–650 pounds
Roasted bran	25–100 pounds
Sugar or coloring	1 quart to 1 gallon
Potato starch	850 pounds
Lactic liquor	1 barrel
Hops	35 pounds
Thick yeast	37 pounds
Table salt	5 pounds
Carbonic acid gas	75 pounds

The ingredients may be varied accordingly to produce a change in color and its flavor may likewise be altered. No malt is used throughout the entire process.

Buel patented (10) a process consisting in pasteurization of raw milk followed by inoculating the milk with lactobacilli. The bacterial count of the milk after inoculation should be between 500 to 10,000 bacteria per cubic centimeter.

Rogers claims (11) a process of making a food product consisting in precipitating the casein of skim milk by the growth of Lactobacilli, breaking the curd by forcing the soured skim milk through a homogenizer and condensing the skim milk to a powder.

Rettger and Cheplin claim (12) a sour milk product containing *Lactobacillus acidophilus*. This product has been used mostly as a medicinal agent (13).

Heuser patented (14) a process for the manufacture of an unfermented beverage made without malt from grain materials such as bran, gluten or spent grains. A thick fluid grain mixture is inoculated with a pure culture of *Lactobacillus pastorianus*, some salt is added and the mixture incubated at about 30°C. The mixture is stirred and allowed to ferment until an acidity of about 0.3 per cent is reached. A sufficient quantity of hot water is added to reduce the acidity to 0.2 per cent and the temperature is raised to 64°C. to prevent further bacterial action. The liquid portion is now separated and clarified and some corn sugar added to give a body to the finished product.

The organism used in this fermentation has an optimum temperature range from 27° to 33°C. It forms acid in dextrose, levulose, galactose, arabinose, maltose, lactose, sucrose, dextrin, raffinose, trehalose and mannitol. It forms 1.5 per cent acidity due to the production of lactic, formic and acetic acids. In this fermentation we have an example of a fermentation by a lactobacillus at a temperature lower than that usually required by other species of the same genus.

In 1916, Fonyo patented (15) a process of preparing grape juice by expressing the juice from the grapes and collecting it in a thermally controlled vat heated to 55°C. The juice is inoculated with a pure culture of *Lactobacillus delbruckii* or other lactobacilli. Lactic acid fermentation proceeds at this temperature but alcoholic fermentation is precluded. The lactic fermentation requires six to eighteen hours during which time the acidity rises to from 0.3 to 0.8 per cent, depending upon conditions of fermentation. When the desired acidity is reached (0.1 to 1.0 per cent) the juice is heated to 82°C. to kill the

Lactobacilli and prevent further increase in acidity. It is advisable to cool the juice to 4.4°C. and to separate out the resulting precipitate by filtration. Since the optimum temperature of *L. delbrucki* is about 35°C. the temperature specified in the process could be carried out to better advantage by using one of the other species of Lactobacilli.

Richard, in 1922, claims (16) a new hygienic food made by fermenting fruit juice with *Lactobacillus bulgaricus*. Suitable fruit juices are pineapple juice and prune juice. Sucrose, glucose or lactose may be added to the juice previous to fermentation. Fermentation is carried out at about 43°C. The acid product may be incorporated in other food compositions or dessicated.

Snelling, in 1923, claims (17) the process of fermenting fruit juices with lactobacilli of the Bulgaricus group. The fermentation proceeds at a temperature in excess of 40°C. for from twelve to sixty hours. Bacteriologically there is no difference between the process of Richard and the later process of Snelling.

TABLE 18

FOUR TYPES OF LACTOBACILLUS BULGARICUS ON BASIS OF CARBOHYDRATE FERMENTATION

TYPE	MALTOSE	DEXTROSE	SUCROSE	RAFFINOSE
A	−	+	−	−
B	−	+	−	+
C	−	+	+	−
D	−	+	+	+

Lactobacillus bulgaricus according to Rahn (18) can be separated into four types on the basis of carbohydrate fermentation (see table 18).

The organism forms from 2.7 to 3.7 per cent lactic acid in milk. The lactic acid formed is either optically inactive or laevo-rotary. A small amount of volatile acid is also formed. The organism is microaerophilic with an optimum temperature between 40° and 45°C. Snelling treats a portion of his substratum with yeast and with *Acetobacter acetus*. The optimum temperature of this fermentation is about 30°C. This acetic portion is added to the main portion which has been subject to lactic fermentation. After mixing the two portions, some fresh juice, water and essential oils are added.

A palatable beverage prepared from tea leaves by the symbiotic action of *Acetobacter xylinum* and Pombe yeast has been described by Arauner (19).

Brown (23) found that fermentation takes place in a pickle brine up to 20 per cent salt concentration. The acidity is due to the formation of lactic, acetic, propionic, butyric and benzoic acids. The gas analysis showed about 90 per cent carbon dioxide and a trace of methane and residual nitrogen. A large part of the gas is formed by surface growth of yeast cells, Torulae, which attack the lactic acid. A trace of alcohol is found.

In an investigation of film-forming fungi isolated from olive, pickel, and other brines and from fermenting or fermented apple juices and California grapes, a pink Torulae, a Mucor and three species of Penicillia were isolated (25). All the organisms grew in nutrient sucrose, dextrose, maltose, lactose, mannite, glycerin, acetic acid, citric acid, lactic acid, malic acid, oxalic acid and tartaric acid, usually without gas formation. Oxalic was the most toxic of the acids. The Mycodermas grew in relatively high acetic acid solution having a pH value of 3.5 and in salt concentrations up to 20 per cent. Sodium benzoate, sulphur dioxide and hydrofluoric acid were the most efficient and practical antiseptics for factory use. A process (26) for curing ripe olives after the preliminary brine fermentaton has been patented.

ACID CURD CHEESE

Whole milk when allowed to sour or when inoculated with a starter permits the separation of the curd. The curd requires no ripening and is in reality a thick sour milk although it is generally known as an acid curd cheese. Cottage cheese is now being made on a commercial scale by many companies. The souring depends upon the normal flora of the milk as does also the flavor. A bitter taste is due to the action of proteolytic organisms present in the milk. Diehm (20) isolated a *Streptococcus lactis* as the predominating organism with two species of Oidia, *Oidium lactis* and *Oidium asteroides*. These organisms were doubtless introduced in the starter.

The following are the characteristics of the genus Oidium:

Square ended rods which form in branching chains, non motile, Gram positive.

Agar colony: Circular, ciliate margin, radiate structure, white, powdery.

Agar slant: Filiform, ciliate margin, white, powdery, raised, opaque.

Gelatin: Mycodermal surface growth. Slowly liquefied if at all. Colonies extend into medium.
Litmus milk: Varies from acid to alkaline.
Potato: Same as any plain agar but more abundant growth.
Indol: Not formed.
Nitrates: May or may not be reduced.
Starch: Not hydrolyzed.

While it is generally assumed that commercial starters are cultures of *Streptococcus lactis* other lactic acid organisms are often present. These additional organisms are largely responsible for the development of the flavor and aroma of the finished product. Symbiotic action frequently occurs which enhances the development of lactic and smaller amounts of volatile acids.

RENNET CURD CHEESE

In addition to the acid curd cheese discussed above, there are a number of varieties of rennet curd cheese that are at times made on a very large scale and may be properly considered as industrial biological problems. There is much similarity in the first stages of manufacture of most of these, the milk being more or less "ripened" by holding in bulk at 30° to 32°C. until sufficient acidity develops, usually 0.2 or up to 0.23 per cent acidity as lactic acid. This process may or may not be hastened by the addition of a starter culture of lactobacilli or *Streptococcus lactis.* Then follows the curding with rennet, cutting the curd when well set, draining off whey, salting, and extracting excess water by pressure.

Those products classed as hard cheese, such as American, Cheddar, and Swiss or Emmenthaler contain under 40 per cent moisture. They must be held for a ripening period, preferably under conditions of controlled temperature and humidity. During this period the casein is acted upon to some extent by the proteolytic enzymes of the lactobacilli and the lactose and lactates present are fermented to produce various volatile fatty acids responsible for the flavor and aroma. In the ripening of Swiss cheese the propionobacters play an important part.

Colon group organisms in the raw milk produce gassy cheese and *Oidium lactis* and spore formers may produce bitter or slimy cheese, so clean milk is essential. Probably the best and quickest test of the quality of milk received at a factory is the methylene blue reductase test which will exclude very bad milk in a half hour period.

In performing the test 0.5 cc. of 2.5 per cent saturated alcoholic methylene blue solution in water is added to 20 cc. of milk warmed to 45° to 48°C. in a sterile test tube. The tube is plugged with cotton, mixed well and held in a water bath at 38° to 39°C. until decolorized. Good clean fresh milk should remain blue for over five hours, and very bad milk may be decolorized in twenty minutes or less.

The soft cheese group including Roquefort, Stilton, Gorgonzola, Brie, Brick, and Limberger contain more moisture and are ripened by the penetrating action of molds, oidia, or bacteria and their products, these organisms settling on the surface of the smaller loafs during storage. The particular organisms responsible for particular flavors are usually prevalent in the storage rooms.

No detailed discussion of cheese microbiology, however, will be given here as it is discussed fully in other works such as Marshall, Thom and Hunter, and Tanner.

MANUFACTURE OF BREAD

In the manufacture of leavened bread the amount of yeast normally employed may be reduced one-half of the usual quantity by subjecting the milk first to proteolytic fermentation. The process was patented (21) in 1917. A portion of a pure culture of *Bacillus subtilis* is transferred to the milk to be used for bread making. This milk is preferably pasteurized or sterilized before inoculation with the pure culture. After the addition of a small amount of salt and baking soda the milk is incubated at 30°C. for about forty-eight hours or until the digestion is complete. Other organisms that are suitable for the fermentation are *Bacillus mesentericus* and *Bacillus mycoides*.

Presumably in the digestion of the protein constituents the nitrogen is made available as a yeast nutrient which accelerates the yeast fermentation. Other nitrogenous products such as beans, peas, cheese, cocoanut press cake when digested so as to render the proteins soluble may serve as a supply of nitrogenous food for the yeast cells.

Coulson describes a milk treated wheat flour and claims (22) a baking flour comprising the combination of cereal flour, 3 to 5 per cent of solids of curdled skim milk and 2 to 5 per cent solids of butter milk. The solids are the residue left after the curdled milk or buttermilk is dried. These nutrients aid in the yeast fermentation of the dough.

Wahl claims (24) a concentrated yeast food and bread improver consisting of lactic acid salts and lactobacilli. The lactic acid mash is

neutralized as the acid is formed and this is transferred to the yeast mash. The yeast cells utilize the lactates which stimulate their growth.

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CHAPTER 28

Meats, Nitrogenous Concentrates, and Eggs

MEAT CURING

In a series of three patents (1, 2, 3) Kurk claims a process for the curing of meat in the presence of nitrates. The selected type of bacterium "which has not previously been isolated nor described" is identified by the following characteristics:

1. It is non-putrefactive.
2. It is non-pathogenic.
3. It is nitrate reducing.
4. It is a micrococcus.

Such a description raises in the mind of a bacteriologist the question as to the value of such a patent. In 1921, the date of issue of the patent, there appears to have been no use made of the descriptive chart for the identification of bacterial species recommended at the 1917 meeting of the Society of American Bacteriologists. The simple question raised is what microörganism is described as being used in the process, for herein lies the success or failure of the process, as specified in the patent claim. A large number, indeed, of the micrococci are non-pathogenic, many are non-putrefactive and nitrate reduction is not an important criterion of the genus. Perchance this organism is *Micrococcus phosphorescens* of Cohn described, in 1882, as isolated from meat in refrigerators, butcher shops and markets. We raise this question merely to emphasize the necessity of a complete bacteriological description of the causative agent in all bacteriological procedure and particularly in the case of a patent claim such as we have here. It should be an added protection to the patentee to completely describe his organism. At the present time numerous organisms are used in different biological processes as different species because the description given in the patent claim is not complete. As bacteriologists we do not know what was patented.

NITROGENOUS CONCENTRATES

Antiseptic substances that readily can be eliminated, such as carbon tetrachloride, permit yeast cells and other microörganisms producing

enzymes to be autolyzed. Kahn patented (4) a process for the manufacture and extraction of nitrogenous products from substances of vegetable origin containing a proteolytic ferment. Compressed yeast is mixed with equal parts of water and from 0.5 to 1 per cent of carbon tetrachloride is added to the mixture. The mixture is digested for about fifteen days, filtered through tannin or charcoal and dried in a current of air, warm enough to remove the antiseptic. The resultant paste is palatable, rich in nitrogenous matter and suitable for making a broth or may be combined with other foodstuffs. The digestion of vegetable products rich in starch necessitate a means of allowing the escape of carbon dioxide gas. Kahn suggests the following antiseptics as suitable.

1. Hydrocarbons.
   Benzene, toluenes (toluols), xylenes and liquid homologues.
2. Chloruretted halogenous derivatives.
   a. Fatty series.
      Chloroform, carbon tetrachloride, dichlorethylene, trichlorethylene, tetrachloracetylene, pentachorethane, symmetrical and unsymmetrical dichlorethanes, chlorides of propyl, butyl and liquid homologues, chlorides of propylene, butylene and liquid homologues.
   b. Aromatic series.
      Mono, polychloro, bromobenzenes and homologues, benzyl chlorides, homologues and their nitrated derivatives.
3. Alcohols.
   Butyl, isobutyl, amyl and higher alcohols.
4. Sulphuretted derivatives.
   Sulphide of carbon, thiocarbonates, mercaptans, fatty sulphides, sulphuretted natural mineral oils (oils with ichthyol) and isosulphocyanic ethers.
5. Aldehydes.
   Formaldehyde and its product of condensation with ammonia, hexamethylenetetramine, acetaldehyde, chloral, acroleine, crotonic aldehyde and furfurol.
6. Ketones of the fatty series or their mixtures.
7. Phenols.
   Phenol, cresols and homologues pure or mixed, halogenous, nitrated, alcoholated, etc., derivatives.
8. Fluorides and bromides.
9. Aniline and its homologues, quinoleine and its homologues.

The antiseptic substances are used singly or mixed with one another. Many of the antispetics are not as easily eliminated from the final product as in the case of carbon tetrachloride and are decidedly toxic.

In a later patent (5) Kahn describes a process for the autolysis of a fish maceration to produce a nitrogen-containing product. In Indo-China a liquid food "nuoc-mam" is prepared by repeated maceration of fish in salt or sea water. Salt in the concentrations used by the Indo-Chinese manufacturers (about 25 per cent) prevents bacterial decomposition but reduces the efficiency of the autolysis of the fish flesh and requires a relatively long period of time. In Kahn's process the fish to be treated are maintained at a temperature between 37° and 55°C. with the addition of only 5 to 10 per cent of salt, by weight or the fresh unsalted fish is maintained between 50° and 55°C. Bacterial infection is prevented but the autodigestion is extremely rapid and the liquid food is produced in one to five days. At the completion of autolysis the fish oil collects on the surface of the liquid. The liquid portion is separated and flavoring or salt is added to the product to suit the taste of the consumer. The residue is suitable as a tanning product or with the oil extracted as a manure. The hydrogen ion concentration of the brine likely determines the rate of autolysis. Since the percentage of salt added is based on the weight of the fish it it hard to determine the per cent of salt in the brine. Ordinary salt does not prevent bacterial growth in concentrations less than about 20 per cent and some species have been isolated from the pure salt itself. We include this process because the material is so readily subject to bacteriological decomposition if the pH value is greater than 5.0.

Balls patented (6) a food product made from fresh yeast. The method consists in separating the yeast cells from the liquid medium by pressing, settling or centrifuging and then heating under pressure until the cells are disrupted and the mass entirely liquefied. The excess moisture is removed in an evaporator or on a water or steam bath until the desired consistency is reached, usually a water content of from 30 to 45 per cent. By autoclaving yeast the flavor and odor is entirely removed producing a tasteless material containing practically all the food value of the original yeast and to which salt, spices or any desired flavoring substances may be added. The usual procedure is to mix 50 grams of compressed yeast in 350 cc. of water and then heat the suspension in an autoclave at 150°C. for about forty-five minutes. The resulting liquid is evaporated to a volume of about 25 cc. The final product is a brown pasty mass giving an opalescent milky liquid when mixed with water. Such a preparation may serve as a base for the preparation and manufacture of other food products.

In 1929, Kahn, Breton and Schaeffer patented (7) a process for treating yeast and mold in the absence of any antiseptic and separating the autolysate from the undigested portion. Attempts have been made to utilize yeasts or yeast concentrates for feeding cattle and other animals. The bacon of animals fed on fresh yeast is of a poor quality and in addition the preparation of yeast concentrates are expensive processes as compared to other sources of nitrogen. Their process consists in the autolysis of yeast cells or other amylonyces (*Mucor rouxii, Mucor oryzae, Aspergillus oryzae, Rhizopus oryzae,* etc.) under controlled conditions. The organisms as taken from the fermentation vat are pressed or dried in a centrifugal machine and emulsified in 0.1 to 0.5 per cent alkaline solution. They are again pressed and washed with dilute hydrochloric acid to produce an optimum hydrogen ion concentration. A third pressing leaves the yeast mass containing approximately 80 per cent of water. It is then placed in large thermostatic vats at a temperature between 36° to 44°C. Sodium chloride may be added in not more than 6 per cent concentrations. The temperature is maintained constant for from three to twenty days until autolysis is sufficient. Agitation and aeration of the vats vary with the particular operation. When autolysis reaches the desired state the contents of the vats are diluted with water in order to facilitate separation of the liquid from the solid portion. The liquid may be bleached and concentrated under vacuum (20 to 40 mm. mercury) at 70°C. The resultant paste contains about 20 per cent water. The pastes thus prepared are suitable for cattle food as well as for human consumption. They may also be mixed with extracts of vitamins A, B, or C.

The patent also suggests the possibility of using the cellulosic residue present in the solid portion of the mash. This portion is heated to 50°C. and greases at a temperature between 40° and 80°C. are thoroughly mixed throughout the residue. The greases may be pure glycerides or mixtures containing fatty acids and soaps. Antiseptic substances are added to prevent decomposition. The resultant product is sold as an artificial composition for the treatment of leather. This by-product composition reduces the expense of the production of the autolyzed liquid.

EGGS

Eggs are decomposed by the action of bacteria. The keeping quality of an egg depends largely upon sanitary conditions and the

care in handling the product. The preparation of dried and frozen eggs is a fast growing industry in China while the preparation of frozen eggs is becoming a more extensive industry in the United States. The preservation of shell eggs in cold storage, however, is one of the most important problems in food economics. A satisfactory method for preservation of shell eggs requires an extensive study of the microörganisms involved and the chemistry of the egg shell and membrane.

Shrinkage, due to loss of carbon dioxide and moisture, is the greatest economic factor encountered in the cold storage of eggs (8). Swenson and Mottern studied the oil absorption of shell eggs to develop a method of oil processing that prevents shrinkage and also replaces the carbon dioxide lost by vacuum control. The mineral oils were colored with Sudan IV to facilitate microscopic observation of penetration. No difference in absorption was found between the shells and membranes of brown eggs and those of white eggs. It was found that eggs dipped in 2 per cent aluminum soap oil under 50 mm. of vacuum at 40°C. for one minute and stored at 37°C. for ten days lost only 0.5 per cent of their weight.

CLADISPORIUM S.

Cladisporium S. better known as the pin point mold of shell eggs belongs to the family Dematiaceae. Its cell walls are dark or smoky. The colonies at first appear dark with an indication of green coloration but the older colonies develop a characteristic moss green, gray-brown pigmentation. The conidiophore branches at the tip with separation of two celled conidia. The original strain was isolated from shell eggs and has contributed to, if not responsible for, the deterioration of eggs in cold storage. It is because of its peculiar cultural characteristics that it is mentioned in this work. It grows on egg shells at normal cold storage temperature for storing shell eggs provided the initial germination takes place before the eggs go into storage. It, also, grows quite well in ordinary mineral oil as demonstrated by T. L. Swenson in his work on the preservation of eggs. It produces a small amount of acid from xylose, levulose and dextrin. On sucrose it produces marked amounts of oxalic acid with no gas liberated. The other carbohydrates are not fermented at ordinary temperatures. Gelatin is generally liquefied.

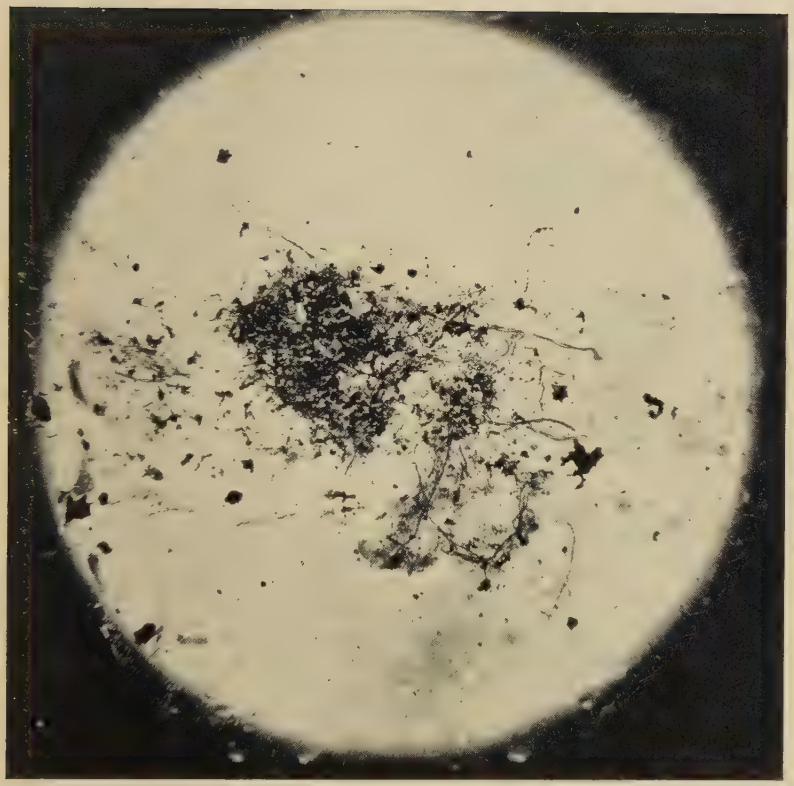

PLATE 2. *Cladosporium S.* Growing in mineral oil. Plate 1 magnified.
(Courtesy of Bureau of Chemistry and Soils, U. S. D. A.)

PLATE 3. *Cladosporium S.* Growing on shell eggs in cold storage. Temperature 33°F., and a relative humidity of 80 per cent.

(After Swenson and James, Bureau of Chemistry and Soils, U. S. D. A.)

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CHAPTER 29

Refrigeration, Apparatus and Filters

REFRIGERATION

The refrigeration industry is of great economic importance and an outstanding example of the combined contributon of engineering and bacteriology. The use of new refrigerants has established this industry from the large scale cold storage plants to the household units with any desired temperature for food preservation. It must be emphasized, however, that low temperatures will not safeguard contaminated materials. The foodstuff is the important factor in food storage for it is kept approximately in the original condition. Good food, prepared under sanitary conditions will readily be preserved by cold storage methods.

Cold prevents the growth and multiplication of living cells and retards the activity of elaborated enzymes. Few microörganisms can grow at O°C. although they quickly decompose organic material that has been subject to refrigeration when it is removed from storage. The comparatively few psychrophillic bacteria are strongly proteolytic, producing an alkaline reaction in litmus milk. They belong to the genus Poseudmonas and are widely distributed in nature.

The optimum temperature for refrigeration depends upon the particular food. Meats, fish and poultry are generally frozen and stored at temperatures from $-4°$ to $-16°$C. Fish and poultry are often stored in the undrawn condition although if the material is skillfully drawn so that care is taken to prevent the tearing of the intestinal tract the drawn material will hold up satisfactorily. The prime requisite is the preparation of a sanitary product for storage. The soiling of the flesh with microörganisms from the intestinal flora insures an initial inoculum for microbial decomposition during the thawing out process. Refrigeration is one of the simplest and best methods of preserving perishable food materials against the action of microörganisms; however, such food should never be passed off for the fresh or chilled material. Generally speaking nothing detrimental is added to the food and no nutritive value is lost in refrigeration of fresh wholesome foodstuffs.

In the storage of fruits and vegetables the temperature should be slightly above zero and the relative humidity should be about 60 per cent saturation to prevent evaporation as much as possible without enhancing mold growth. Milk and other dairy products are best preserved at the same temperature.

PURE CULTURE APPARATUS

Magne describes (1) a pure culture apparatus for yeast, mold and bacteria that can be used to inoculate large scale operations. The system is one of transfer from a small initial vat to a larger secondary vat.

CATAPHORESIS

Shemitz and Wechsler have patented (2) an apparatus and process for detecting and segregating bacteria in liquids by means of cataphoresis.

THE CATADYN METHOD

The Catadyn method of water sterilization will remove non-spore forming rods in two hours. The germicidal effect is due to the metallic ions formed by accumulation of molecular oxygen on various metals such as silver and copper (5).

FILTER MEMBRANES

A method of preparing a filter for separating bacteria and colloidal particles from a medium in which they are suspended was patented (3) in 1922 by Zsigmondy and Bachmann. The filters are essentially cellulose esters as nitrocellulose or cellulose acetate. An example requires 10 parts, by weight, of dried nitrocellulose dissolved in 80 parts of acetone and 37 parts of glacial acetic acid. The solution is poured on a glass plate and allowed to dry at about 18°C. in air with a 60 per cent relative humidity. The permeability of the membranes can be controlled by varying the percentage of cellulose esters in solution, the composition of the solvent mixture or the relative humidity in the evaporating space.

FILTER BODY

A process for forming a filter body for the removal of bacteria from liquids was patented (4) in 1918. It consists in mixing infusorial earth, flax and cotton with formalin to form a paste and then filtering

the mass, by drying, at 60°C. The filter body is sterilized at 120° to 150°C. when ready for use.

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Section IX

THE HYDROCARBONS

CHAPTER 30

Coal, Petroleum and Phenols

COAL

The action of bacteria on coal was reported by Galle (1) in 1910. Small amounts of methane, carbon monoxide and carbon dioxide were formed. A *Micrococcus carbo* and *Micrococcus lignitum* have been grown on a wood charcoal medium at about 20°C. Pulverized coal incubated at 37°C. has also been used as a culture medium. Some bacilli were observed and designated as *Bacillus nacraceus*, *Bacillus subtilis*, *Bacillus mesentericus* and *Bacillus pseudosubtilis* as well as Sarcina and other forms.

Methane forms about 35 per cent of coal gas and serves as a source of energy for *Methanomonas methanica*, a short rod which oxidizes it to carbon dioxide and water. Carbon monoxide is oxidized by a small, non-motile rod, *Carboxydomonas oligocarbophila*, to carbon dioxide. This organism grows best at 25°C. In these oxidations we have a good example of the diversified material from which the microbial population can derive energy. While in the case of animals the food supply is largely limited to plant and animal tissue, the bacteria utilize many gases as food.

PETROLEUM

The belief that petroleum has been produced from the microbial decomposition of organic vegetable matter complies more fully with views of geologists than do the numerous chemical hypotheses. The optical activity of natural petroleum indicates again a biological genesis (2). Crude oil is found widely distributed over the world but particularly in the United States, Mexico, Russia, Roumania and Persia.

The soil of the Russian oil bearing regions are said to be rich in a great variety of microörganisms utilizing hydrocarbons and displaying considerably specificity. Tauson (3) reports that microörganisms obtained from the soil of the Baku region (Russia) can utilize hard

(m78°C.) as well as soft (m45°C.) paraffins or white petrolatum. Conditions necessary for the oxidation of the paraffins are:

1. Presence of water with mineral salts dissolved, the presence of nitrate or ammonia, salts being a matter of indifference except that in the latter case calcium carbonate must be available to neutralize the acid set free by the escape of ammonia.
2. Free oxygen is essential.
3. The reaction of the solution should be neutral.

The microörganisms utilizing paraffin can also oxidize fats and fatty acids. The same author records the names of various types of bacteria and mold. At present we can recognize two genera.

1. Pseudomonas: Soil and water bacteria producing a green, blue or yellow green water soluble pigment. Rods, generally motile with polar flagella, Gram negative. Optimum, 20° to 25°C., aerobic. Type organisms: *Pseudomonas aeruginosa* and *Pseudomonas fluorescens.*
2. Achromobacter: Non-pigment forming rods, motile with peritrichous flagella, Gram negative, aerobic. Optimum, 25°C. Type organism: *Achromobacter stutzeri.*

These organisms utilize paraffins as well as kerosene. Sohnagen (4) has prepared a paraffin agar suitable for growing hydrocarbon destroying organisms.

	Grams
Ammonium chloride	0.5
Dipotassium phosphate	0.5
Magnesium sulphate	0.5
Paraffin oil	10.0
Agar	20.0
Water	1000.0 cc.

A Penicillium can utilize 80 per cent of a soft paraffin. *Aspergillus flavus* and some bacteria can utilize paraffin, plant and animal waxes and higher alcohols. As there is no evidence of increasing acidity in the cultures it is believed that the paraffins are oxidized to carbon dioxide and water. The following groups of hydrocarbons are oxidized: Paraffins, naphthenes (?), olefins and terpenes as well as the aromatic compounds benzene, toluene and xylene.

An organism, *B. benzoli*, is reported as utilizing 8 grams of benzene in about thirty-four to forty-two days in the presence of nitrates. Three types of organisms were able to utilize naphthalene as a source of carbon and other varieties utilized phenanthrene. The fermenta-

tion of the hydrocarbons indicates that crude oil may undergo progressive oxidative destruction in nature and that changes may take place in crude oil before it comes out from underground. Ammonia is usually a better source of nitrogen than the nitrates.

Tausz (5) prepared a medium containing:

Magnesium ammonium phosphate	0.10 gram
Dipotassium phosphate	0.08 gram
Calcium sulphate	0.01 gram
Sodium chloride	trace
Potassium iodide	trace
Ferric chloride	trace
Ditch water	100 cc.

The medium was covered with a 1 to 2 mm. layer of crude petroleum and allowed to ferment several weeks. The liquid became turbid with asphalt formation. It was found possible to separate the paraffin from the naphthene hydrocarbons. Three new species of bacteria were isolated.

PHENOL

The treatment of phenol wastes is a problem that frequently confronts the engineer. Although the determination of the phenol coefficient is the accepted method of determining the germicidal power of a disinfectant it must not be overlooked that some bacteria are capable of using aromatic compounds, as phenol, as a source of energy. The organisms belonging to this genus, Mycoplana, are typical soil bacteria, curved and irregular rods showing branching cells, usually motile, Gram nagative, aerobic, and with an optimum temperature of 30° to 35°C.

Michel (8) found that *Lactobacillus bulgaricus* acquired the property of resisting phenol. Intestinal organisms have been reported as forming phenol from tyrosine by splitting off from the alanine side chain (7).

The purification of waste phenol liquors involving a bacterial fermentation was patented (6) by Davis and Semenow in 1922. The phenol is removed from the liquor by absorption as it passes through a filter bed of finely ground humus material. After the filter bed has been used and its efficiency reduced, it is revived by stimulating the growth of phenol destroying bacteria. The ground lignite or peat is allowed to remain damp and the material is aerated at about 25°C.

Revivification is hastened by inoculation with sewage sludge. According to patent claims the revivified material is as effective as the fresh humus material.

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CHAPTER 31

The Microbiology of Rubber Latex

RUBBER LATEX

Rubber is a hydrocarbon contained in the latex of certain plants. Such plants are characterized by the presence of capillary vessels containing a milky juice in their bark, leaves, stems and roots. Latex is an emulsion varying in composition but contains approximately:

	per cent
Water	51
Rubber	40
Protein	3
Resins	4
Mineral matter	2

The latex is collected from the plants by making an incision into these latex bearing canals and allowing the fluid to pass out into suitable containers. The latex coagulates in varying periods of time depending upon the bacterial flora. Since the market value of the rubber may be influenced by the coagulation of the latex it is essential to control this step in the process of preparation for manufacture.

The principal methods of coagulation are by means of heat and smoke, the addition of chemicals, phenol, alum, sodium silicofluoride, acetic, formic or tartaric acid and by centrifuging. The "spontaneous" coagulation of rubber is facilitated by an acid fermentation of the latex.

MICROBIOLOGY OF RUBBER LATEX

The microbiology of rubber latex is definitely associated with its keeping quality and behavior. Large numbers of bacteria are present and belong to several different groups. The group producing acid from carbohydrates when grown on ammonia free sterile latex produces a putrefactive odor and slow coagulation of the latex itself. The addition of even small amounts of sugars greatly hasten coagulation which takes place in about twenty hours at 37°C., the globules of

rubber coalescing to form the coagulum, and no odor of putrefaction is detected.

The non-acid group does not produce coagulation. Prescott (1) notes eleven types as characteristic of latex. Of these the four strong acid producers did not form a coagulum when ammonia was present. The microörganisms have access to the latex in the operation of collecting and shipping. The spore forming types predominate.

Denier and Vernet (2) state that the spontaneous coagulation, within forty-eight hours, of the milky juice from which rubber is prepared is one of the oldest procedures known in the making of rubber. They have studied the bacterial flora in the latex and have isolated one which is more commonly present than others and which appears to influence beneficially the coagulation. Planted in the latex under proper precautions coagulation takes place within twenty-four hours with no discoloration. Picado (4) also considers the possibility of modifying rubber by pure culture inoculations of latex.

Recently Corbet (3) has described a new species which he names *Bacillus pandora*. It is a spore forming rod, 3.1 microns long by 0.9 microns wide, facultative anaerobe, Gram positive, stains readily with carbol fuchsin, liquefies gelatin, reduces nitrates to nitrites with no gas production, decolorizes litmus milk, does not produce hydrogen sulphide, coagulates milk, forms indol, produces gas in glucose and acid and gas in lactose and sucrose. It is most active in a pH range from 4.0 to 10.0. Albumin is attacked with the liberation of ammonia. The starches or celluloses are not fermented. When grown in latex it produces an acid reaction but no gas is formed and the rubber flocculates in two to three days.

Natural coagulation of the rubber is observed to take place when there is an acid reaction in the bulk of the latex and an alkaline reaction at the surface. This surface alkalinity is due to proteolysis taking place in the latex with the production of ammonia.

At least one species of bacterium has been able to ferment the latex "carbohydrate" (methyl-*l*-inositol, a cyclic compound) with a resulting acid reaction. Sterile latex does not coagulate until inoculated and when coagulation does occur the bacterial count is high.

DETERIORATION OF RUBBER

Sohngen and Fol isolated two actinomyces, *A. elastica* and *A. fuscus*, from garden soil and soil washings that were able to attack rubber.

When rubber films are prepared by evaporating benzol-rubber solutions, after the albumins have settled out, and then treated with tryptic enzymes to remove the nitrogenous compounds vigorous growth of the actinomyces is observed. In about one month the rubber film loses its resilience and shows perforations where the fungi have been most active. De Vries (5) tested the influence of various organisms on plantation rubber, particularly the Penicillia and the Aspergilli, with the following results:

	per cent loss in weight
Unsmoked sheet, 40 days molded	4.0
Smoked sheet, 9 months molded	7.5
Smoked sheet, 19 months molded	13.5

The following shows the effect of fungal growth after nineteen months:

	Infected per cent	*Uninfected per cent*
Weight of rubber and fungi	88.6	97.4
Weight free of mycelium	86.9	97.4
Moisture	3.4	0.5
Total weight loss as dry matter	15.5	2.1

No change in plasticity or vulcanizing quality was observed, but there was some decrease in viscosity of the benzol solution. In a second series of tests the molded rubber showed a 22 per cent loss in weight in two years and a 30 per cent loss in weight after five years of storage.

Blommendaal (6) isolated micrococcoid slime bacteria from crepe rubber showing distinct white spots. These organisms were found to occur whenever moist coagulum, scraps or lumps, was stored in the open air. Infection may occur from slimy cement floors. As control measures he recommends storage of coagulum under water, and the smoking of crepe as quickly as possible.

We have noted such white spots in baled crepe rubber in warehouses in this country and during transport. It is rather generally conceded however, that the presence of mildew, while undesirable, does not affect the usability of the rubber in the various manufacturing processes.

The manufacture of a rubber substitute by a fermentation process is covered by a British patent (7). Seaweed is treated with dilute hydrochloric acid, washed with alcohol and mixed with equal parts of

candleweed, or desert milkweed, cactus juice, manila gum, gum arabic, gutta horfoot, Indian hemp gum, glue, fish oil, sunflower oil, bastard gum, shellac, neen insect gum, cativo gum, sharks' eggs, gum chicle or balanta. To such a preparation an equal amount of starch is added and the mass fermented with *Clostridium butyricum.* The two species designated in the patent claim are likely the same organism. The fermented mass is cooled and resin or fish oil is added as well as petroleum products, a solution of sulphur, casein in ammoniacal solution with heating to 100°C., cooling to 35°C., and adding milk of lime followed by carbon dioxide gas and oil.

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Section X

COMMERCIAL ENZYME PRODUCTION

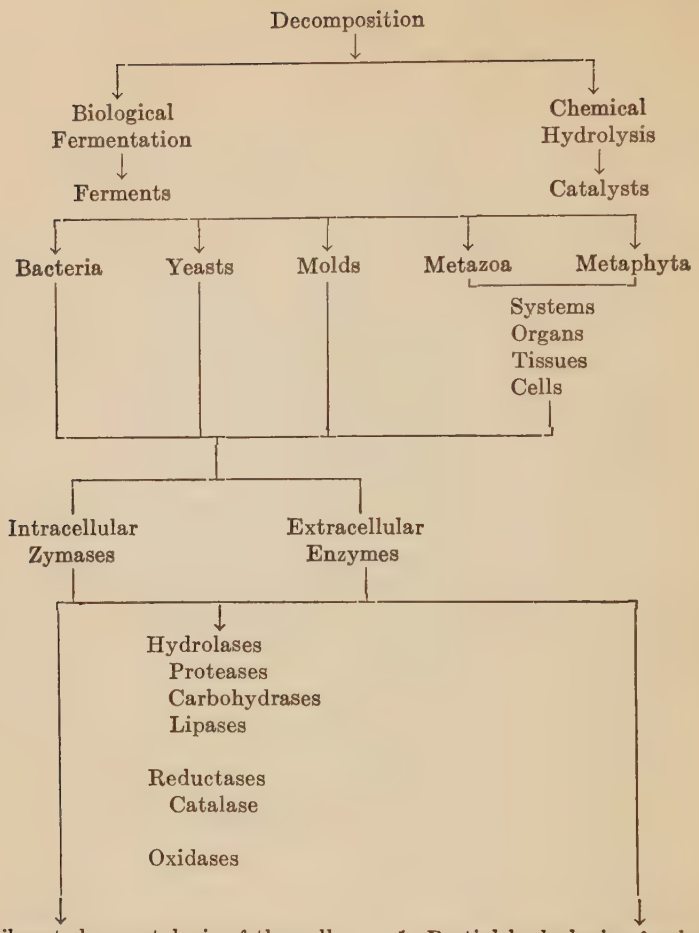
INTRODUCTION

The term fermentation generally defines any chemical change brought about by microörganisms. This change is directly the result of enzyme activity. The enzymes are elaborated by living cells and are characterized by being either intracellular or extracellular. The intracellular enzymes are definitely related to the cell metabolism and fermentations of this nature are heat producting whereas the extracellular enzymes are readily diffused through the cell membrane where they react on the substrate to prepare nutrients that are suitable for absorption. Enzymes generally are inactivated at a temperature slightly above the thermal death point of the vegetative cell.

Boidin and Effront (1) have patented a method of producing bacterial enzyme preparations for industrial uses. Worts or other media rich in assimilable nitrogen may be peptonized by treatment with cultures of proteolytic bacteria as set forth in an earlier patent. A clear wort is preferable for subsequent growth of the desired organisms. When the medium is made by using ammonical salts a small amount of sugar, starch or other carbohydrate should be added as a source of carbon. When using organic nitrogen, peptone, the carbohydrates are not necessary in the medium. A medium suitable for the production of bacterial or mold enzyme is made by using:

	grams
Potassium chloride	1.50
Sodium chloride	0.50
Potassium sulphate	0.50
Calcium tartrate or citrate	3 to 10
Magnesium tartrate or citrate	3 to 10
Potassium or sodium phosphate	3 to 10
Ammonium carbonate	0.50
Iron sulphate	0.10
Manganese sulphate	0.10
Ammonium sulphate	5 to 15
Glucose or starch	25 to 35
Water	1000 cc.

An alkaline organic medium can be prepared by using vegetable juices and a small amount of iron and manganese salts. Dissolved



1. Liberated on autolysis of the cell.	1. Partial hydrolysis of substrate.
2. Heat produced during fermentation.	2. Little or no heat produced.
3. Cell derives energy from fermentation.	3. Cell proper derives no energy.
4. End products a result of cell metabolism.	4. Incorporated in substrate.

Activators are inorganic agents that enhance fermentation.
Kinases are organic agents stimulating the enzyme to activity.

FIG. 12. DIAGRAMMATIC REPRESENTATION OF BIOLOGICAL AGENTS BRINGING ABOUT DECOMPOSITION

proteins can be used as when peanut oil cake is mixed with water and inoculated with proteolytic organisms. The material is allowed to ferment about thirty-six hours at 55°C. at which time it is filtered and neutralized. The filtered wort is cooled and then inoculated with the required organisms. The same workers illustrate an apparatus suitable for their process.

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CHAPTER 32

Amylase Production

INDUSTRIAL USES

At present diastase is used in the textile industries to remove excess size from woven fabrics, for de-gumming silk, and in the canning industries to clarify jellies by dissolving out amylaceous materials introduced with the pectin (30). Boidin and Effront (1) have patented a process for freeing textile materials from size by the action of bacterial enzymes. Bloede (2, 3) patented two processes for the manufacture of vegetable glue by hydrolyzing the starch with bacterial enzymes until the desired consistency is obtained. Dox (23) argues against the specificity of fungus enzymes.

MALT DIASTASE

A fermentation process for the manufacture of pure diastase from malt by the removal of the carbohydrate and nitrogen containing substances with yeasts living in symbiosis with lactobacilli is claimed in a patent by Frankel (4). Pale, kiln dried malt is mashed in water and the mash separated as quickly as possible from the grains. The wort with the addition of pressed yeast and some calcium carbonate is allowed to ferment. The entire fitrate which must not contain any reducing sugars is concetrated in vacuo, until the calcium lactate starts to crystallize, after which the highly concentrated solution is left to crystallize out in the cold.

Pollak (5) claims the addition of a small amount of a strong reducing agent to the diastase solution to prevent accumulation of the lactobacilli. He suggests the use of sodium thiosulphate in 0.3 per cent solution. In a later patent (6) he neutralizes lactic acid with calcium carbonate so that the malt can be successively extracted for the separation of the diastase.

ORGANISMS

Diastase is formed by many different microörganisms. The commercial product is produced chiefly by a few as: *Aspergillus oryzae*,

Aspergillus niger, Penicillium glaucum, Mucor rouxii, Bacillus subtilis, and Bacillus mesentericus.

The following classification and description of *Mucor rouxii* is condensed from Lafar

Zygomycetes—Fungi capable of producing zygospores.
Mucoraceae—Sporangial fructification—no conidia.
Mucoreae—One type of sporangium-sporangium remains attached to sporangiophore after rupturing.
Mucor—No spiny branches on suspensores, sporangiophores with silky gloss, liquefaction of ripe sporangeal membrane

Mucor rouxii: Sympodial branching of the sporangiophore. Sporangiophore, 1 mm. by 7 to 14 microns, upright or declinate, sporangia globular, glass clear and yellowish when ripe, up to 50 microns in diameter. After rupture considerable smooth colorless membrane left as a collar on sporangiophore. Columella globular, slightly flattened, smooth, colorless 23 to 32 microns by 20 to 28 microns. Spores colorless, generally elongated 5 to 2.8 microns, rarely globular. Completely fill the sporangium. On sugar-agar often incompletely developed. Mycelium produces an abundance of gemmae, globular or oval, colorless or pale yellow to light brown with thick smooth colorless membrane, 10 by 100 microns. Resting cells seen in Chinese yeast. On solid media mycelium orange colored, due to golden yellow drops at room temperature. No coloration at 40°C. Optimum temperature, 35° to 40°C.

BACTERIAL ENZYMES

Boidin and Effront (7) patented a process for treating amylaceous substances with bacterial enzymes that are definitely diastatic. In a French patent (8) the same workers covered the action of liquefying bacteria, amylolytic and proteolytic, using organisms such as *Bacillus subtilis* or *Bacillus mesentericus.* The maximum lytic power was obtained by repeated cultures of the microörganisms on raw material strongly nitrogenized and relatively poor in carbohydrates under aerobic conditions at about 35°C., and with abundant pellicle formation. The starch is hydrolyzed only in a slightly alkaline solution. In a later patent (9) the same investigators claim that to obtain amylolytic or proteolytic enzymes from bacteria it is necessary to cultivate them in direct contact with air at the surface of the medium. This results in an alkaline reaction of the medium with increased enzymes production. In a series of ninety-six-hour cultures grown on a 10 per cent soy wort the following hydrolyzing powers were obtained.

100 in glass
25 in galvanized iron

33 in tinned iron
25 in tinned copper
14 in iron
12 in copper
3 in copper in thin sheets
100 in enameled metals
100 in aluminum

Bacterial enzymes are active up to 85°C. although most rapid hydrolysis occurs around 65°C.

DIASTASE

Industrial diastase produced by molds has been used in the oriental countries for centuries, especially in Japan. The diastase is sold in the form of rice batches fermented by *Aspergillus oryzae* and associated species. It is used like malt in the occidental countries. The amylo process has been used extensively in the alcoholic industries. In 1891 Takamine (11) applied Aspergilli preparations to the American brewing industry, with partial success, however the process has been studied and greatly improved since that time. In Japan in 1912 the total cost of fungus products aggregated more than $200,000,000. In 1875, Kozai (12) made a review of the literature, indicating the amount of work already done in this field.

The mold used in the Japanese fermentation was not known until 1876 when Ahlburg isolated it and called it *Eurotium oryzae*. In 1883 Cohn called the genus Aspergillus. In 1878 Korschelt published the first description of Japanese rice saccharification and isolated the enzyme concerned to which he gave the name eurotin (13).

Takamine has patented (14) a number of processes for the manufacture of diastase by the use of *Eurotium oryzae*, Aspergilli, Mucors and Penicillia. The earliest of these, 1894, suggests the use of bran as a substratum because of its loose and coarse nature providing a large surface for growth as well as its cheapness, constancy of composition and the fact that it is rich in nutrients for the microörganism. In a later patent, 1913, he claims acclimatizing the spores to various antiseptics, preferably formaldehyde, so that bacterial growth will not inhibit enzyme formation. The mold is grown on 1:3000 formaldehyde for five generations and then the concentration increased until 1:1500 is reached. He produced commercially in his laboratories two preparations, one solid called taka-diastase, and the other

a water extract of the enzyme called polyzime which contains other enzymes.

The mold itself produces on artificial media a luxurious yellow-green mycelium with numerous closely packed conidiophores. Each conidiophore terminates in a spherical or clavate globule, with a single row of radially arranged sterigmata. It grows well on Czapek's medium.

The amount of any particular product which is elaborated by the mold depends a great deal upon the substratum upon which it is grown. Funke (15) has found that molds previously grown on starch media produce more diastase than molds grown on starch poor media. In other words the mold may be educated to produce more enzyme. Different strains of the same mold produce varying amounts of the same enzyme. A single strain may be high in diastase, the opposite or high or low in protease (13). In other words there is no correlation between the amounts of different enzymes produced. The addition of a particular substance to the culture medium does not cause production of new enzymes but may increase the original enzyme (16).

According to Oshima and Church (17) substances in the culture medium closely related to the enzymes desired, in chemical structure, appear to be more efficient in the stimulation of the production of that enzyme than substances not so closely related; for example, starch is more efficient than dextrin which is more efficient than maltose, etc. On the other hand Funke (15) working on *Aspergillus niger* finds that the amount of enzyme produced does not vary with the starch or glucose in the medium but that maltose does inhibit its formation. Karrer (28) finds that sucrose stimulates spore germination.

There is general agreement that the growth of the mold is entirely aerobic, with an optimum temperature of 30° to 35°C. and 50 per cent moisture in the substratum. The optimum pH range for enzyme production is a value between 4.8 to 5.2.

The enzyme is most abundant at the time of sporulation after which it decreases slowly. This most abundant period takes place two days after inoculation on bran. The enzyme is formed intracellularly and according to Funke it is excreted into the medium as produced, but Oshima and Church state that it remains intracellular until sporulation begins when it is excreted. The extra and intra cellular enzymes are the same.

PRODUCTION OF DIASTASE

For studying the production of enzyme in the laboratory Oshima and Church (17) suggest that the mold be grown on wheat bran in small Erlenmeyer flasks with 50 per cent water and incubated at 30°C. for three days, after which it may be extracted with water and the enzyme tested.

Industrially, in the Takamine laboratories, wheat bran was moistened and steamed for sterilization. Then it was cooled to 40°C. and inoculated with a small quantity of spores. The mass was spread in layers 1½ inches thick on cement floors or placed on trays covered with a fine wire netting and arranged in tiers to allow free access of air. These were kept at 30°C. for forty-eight hours to form a heavy sporulating mat and then extracted with water and dried in air to a moisture content of about 10 per cent.

Knapp (18) grew the mold on various culture media, in particular white bread. He found that a small amount of sucrose stimulates spore germination and helps to initiate growth on laboratory medium. Witkin, in the writer's laboratory, had good results with asparagine as the source of nitrogen in preference to inorganic salts.

The enzyme may be purified by precipitation with 70 per cent alcohol, although Sherman and Tanberg (19) found that 60 to 65 per cent alcohol by volume of 95 per cent alcohol precipitates a purer preparation. The latter also recommend precipitation with ammonium sulphate with subsequent dialysis against water and drying followed by fractional precipitation by the alcohol. Sherman and Schlesinger (20) dry the enzyme in a vacuum over concentrated sulfuric acid.

Commercial mold diastase is necessarily a mixture of many enzymes hence the extract has been called polyzime. Waksman (21) finds in *Aspergillus oryzae* the following enzymes: Amidase, diastase, catalase, inulase, invertase, lactase, lipase, maltase, protease, rennet, sulphatase and cytase. Various authors have found it to be from three to six times more powerful in its action than malt diastase. The water extract polyzime has a diastatic power of D40° –thirty minutes equals 3000 on the Wolgemuth scale, which means that 1 cc. of the solution will hydrolyze 3000 cc. of a 1 per cent starch solution in thirty minutes. The power of the powdered preparation using the same scale is a value of 62,500.

Taka-diastase is stable below 40°C. at which temperatures it keeps

indefinitely, but it is destroyed in one hour at 65°C. It may be preserved from bacterial action by any of the ordinary enzyme preservatives. Oshima (24) suggests 0.05 to 0.2 per cent thymol.

TESTS FOR DIASTATIC ACTION

The hydrolysis of starch takes place in two steps, starch to dextrin and dextrin to maltose. The maltose is further hydrolyzed to glucose. The action of taka-diastase is so rapid that it has led at least one observer to the conclusion that the change from starch to glucose is accomplished by one enzyme. His conclusions have not been substantiated. Waksman (21) believes that two separate enzymes are responsible for the change from starch to maltose. He calls these enzymes amylase and dextrinase. Measurement of diastase depends on either of these two reactions. The saccharogenic method depends upon the formation of maltose which is measured either by copper reduction or by following the reaction with the polariscope. The amyloclastic method depends upon the disappearance of the blue color which starch gives with iodine.

Lintner's method (22) consists in testing the reduction with Fehling's solution by a 2 per cent starch solution containing varying amounts of the enzyme. Oshima (24) modified Lintner's method by using 10 cc. of the enzyme extract placed in 100 cc. of a 2 per cent starch solution at 40°C. for thirty minutes followed by adding 10 cc. of N/5 sodium hydroxide to check the enzyme action. Graduated portions were added to 5 cc. of Fehling's solution and placed in a boiling water bath for ten minutes, shaking once, to determine the smallest amount just reducing the alkaline copper solution. Waksman objects to these methods because the end products differ with the different enzymes.

Johnson (25) uses 500 grams of a pure potato starch paste washed repeatedly with distilled water by decantation, collected on a Buchner funnel and dried by spreading on glass plates for three hours in an air current at 50°C., rubbed up in a mortar then held four hours at 80°C. resulting in a moisture content of about 9.5 per cent. Twenty grams of anhydrous starch (22.2 grams of the actual product) is made into a paste with 100 cc. of water. This is poured into 800 cc. of boiling distilled water for ten minutes and made up to 1000 grams, by weight, with distilled water, then heated and shaken. For the test

proper 50-gram amounts are placed in 250 cc. flasks and held at 40°C. in a water bath. The test solution consists of:

	grams
Iodine	2.0
Potassium iodide	4.0
Water	250 cc.

Equal volumes of the starch solution containing 1, 2, 3, 4, 5 and 6 cc. of the enzyme are tested after eight minutes' digestion. To 5 drops of the starch mixture add 5 cc. of the dilute iodine solution. The first rough test should show clear in one flask at ten minutes. Make a more accurate test with 100 grams of starch paste plus enzyme solution containing amounts increasing by 0.4 cc.

Waksman (26) uses 10 cc. of a 2 per cent washed starch paste in large test tubes at 40°C. Clearing indicates the end point. To increase sharpness the starch may be colored with neutral red by treating 50 to 100 grams of dry starch with 100 cc. of 0.5 per cent neutral red and washing until wash water is colorless and dry. A unit of enzyme is the amount that liquefies 10 cc. of the starch solution at 40°C. in thirty minutes or any specified time.

K = concentrate unit

E = enzyme concentrate

T = time

$$K = E \times T$$

In four tests:

if	E = 0.1	0.2	0.4	0.6
and	T = 12.5	6.0	3.0	2.0
then	K = 1.25	1.20	1.20	1.20

Average K = 1.21

If $T = 30$ then $E = \frac{K}{T}$ or $\frac{1.2}{30} = 0.04$

If enzyme solution was diluted 1:10 then $\frac{1}{0.1 \times 0.04} = 250$ units.

A modification of Wolgemuth's method of measuring amyloclastic power is as follows.

1. Add varying amounts of the enzyme to be tested to a series of test tubes.

2. Add 5 cc. of 0.1 per cent soluble starch to each of the tubes starting with the tube of least concentration.

3. Keep at 38° to 40°C. for thirty minutes.

4. Add 1 to 3 drops of N/50 iodine solution to each tube.

The yellow tube containing the highest amount of enzyme is expressed as the number of cubic centimeter of 1 per cent starch which 1 cc. of enzyme will convert in thirty minutes.

Taka-diastase has a relatively higher amyloclastic than saccharogenic power. The ratio is 6:1 to 9:1 (27). In this respect it is just the opposite of malt diastase which has a marked saccharogenic power.

Oshima and Church (17) give a method for the quantitative estimation of protease produced by the mold. The amount of enzyme is indicated by the clearing of skim milk agar, soy bean agar, gelatin, or casein. It is to be noted that amylase and protease are produced at the same time.

The optimum pH value for the enzyme action is slightly in the alkaline range. The optimum temperature is 40° to 50°C. The action is stimulated by phosphates, aluminum salts, asparagine (11), and neutral electrolytes (19). Takamine and Oshima (29) find sodium chloride to have an inhibitory action on the enzyme.

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CHAPTER 33

Pectinase Production

Bourquelot and Herrissey (1) were the first to study pectinase, having prepared a solution in 1898 from malt. Jones, quoted by Erwin Smith (2), was the first to study bacterial pectinase in any detail. He was interested in it as a product of the organism which he discovered as the cause of soft-rot in carrots, now known as *Erwinia carotovorus*. His method of preparing a concentrated enzyme was to grow the organism on a carrot infusion, then add enough alcohol to make the solution 80 per cent by volume. The enzyme and all protein materials were thrown down and were rapidly filtered out, dried with alcohol, then dried with more alcohol, then air at 40°C., and finally over sulphuric acid. Jones reported this concentrate contained fifteen times more enzyme than the best broth culture of his organism. It was quite stable when dry. It was inactivated by mercuric chloride and formaldehyde, but thymol, chloroform, and toluol could be used to preserve the aqueous solution.

Jones reported his maximum enzyme production occurred when the bacillus was grown at 18° to 22°C., in cultures from one and one-half, to five days old. The enzyme acted best in alkaline solutions, and had no diastatic power. He reported that bacterial filters did not pass the enzyme.

Harter and Weimer (3, 4, 5, 6, 7, 8, 9) in a recent series of papers report more studies on this enzyme from the viewpoint of plant pathologists. Their organism was a mold causing softrot in sweet potatoes, *Rhizopus triciti*, and other species of the genus. They did not attempt to prepare a concentrated enzyme, but devoted their time to studying relative production of enzyme by different species of the genus, under different conditions. Their medium was sweet potato broth, made by prolonged boiling of peeled potatoes, followed by filtration. The mold was grown on this at 30°C. for three days, then the mycelium mat was removed, washed with acetone and ether, and ground in sand so as to rupture the hyphae completely. A suspension of the sand and mycelium in water was then tested for enzyme content, and compared with the enzyme in the broth. No absolute measurement of enzyme content were made.

These workers found the maximum enzyme production in cultures from two to seven days old. The mold did not produce the enzyme when reducing sugars were abundant in the substrate, confirming the statement of Waksman (14) that enzymes are not produced when the products they would form are present as sources of nourishment. Harter and Weimer also report that the enzyme is absorbed by filter paper, and is thrown down partially by the centifuge. They found the same mold, under the same conditions, yielded some amylase (a diastase). The presence of this enzyme would be an advantage in retting, since there is a certain amount of starch to be dissolved in fibrous plants, as well as the pectins.

METHODS FOR CONCENTRATING ENZYMES

Some of the general properties of enzymes deserve more attention and there is also the need of developing a method for concentration of an enzyme solution made by bacterial or mold action. Nord (10) suggests that any purification procedure may only serve to separate the enzyme from a possible co-enzyme necessary for its action, and thus render it worthless. Enzymes are colloids, and can be precipitated onto certain other colloid particles, such as alumina and kaolin (11). Using this precipitation at different pH values, a mixture of enzymes may be separated to a certain extent. According to Waksman, enzymes may be precipitated by ammonium sulphate, but require greater concentrations of the salt than do most proteins. This makes it possible to precipitate most of the proteins first, then by increasing the concentration of the salt to precipitate the enzyme. It has been suggested by Sastri and Norris that enzymes may be purified by freezing the water out of their aqueous solutions. Yeast and bacteria may be used commercially, according to a patent (12) to purify enzyme preparations from carbohydrates and proteins. Alcohol or acetone will precipitate the enzyme from aqueous solutions, with little injury, if they are removed rapidly from contact with the precipitate. But in spite of the many ways available for concentrating Waksman states (14) that no enzyme has yet been made in a pure state, even in the laboratory.

MEASURING PECTINASE ACTIVITY

All the literature found dealing with this problem reports measurement of enzyme activity based on the time required for maceration of

discs of raw vegetable tissue. This measurement may be adequate for comparative purposes with weak enzymes, but it does not offer a duplicate test, nor one with the least suggestion of an absolute meaning. While it is an advantage to use a material almost identical with the substratum the enzyme is known to attack, we feel the indefinite composition of the vegetable discs more than offsets this advantage.

Since pectinase hydrolyzes the pectin molecule, and since the nucleus of protopectin of vegetables, and pectin of commerce are the same, it would appear that pectin solutions of jellies could be used for measurement of enzyme activity. Of course no definite statement can be made until experiment had shown that the digestion of soluble pectin paralleled the maceration of vegetables. Such may be the case. Almost all the pectin gels found in the literature require the sugar to be 50 per cent or more of the total weight of jelly. Only one reference suggests a gel without sugar. Griggs and Johnston (13) report that one drop of 0.01 normal lead acetate will yield a firm hard gel with a 1 per cent solution of pectin. A gel can also be formed by the use of pectase as coagulant, but this seems to need sugar and acid also. Waksman gives a method for preparation of a pectase (14), but it is a much less definite compound than a lead acetate solution, so if the latter can be used it is preferable.

Since there are no tests for traces of pectin, it is impossible to note the time required for a given weight of pectin to absolutely disappear from a solution, as can readily be done with starch or casein in assaying diastase or pepsin. However, pectin is hydrolyzed by pectinase to form, among other things, reducing sugars. This points to the possibility of a reducing sugar determination as an estimate of pectinase activity. The details of this must be worked out by more experimentation.

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CHAPTER 34

Protease Production

The action of the proteolytic bacteria has already been discussed. The yeast cells are not actively proteolytic. Many of the molds are able to attack this organic material. Some of the molds produce both an amylase and a protease which are extracellular in matured mycelia and can be prepared for industrial use. A commercial preparation is also made from the fruit of the pawpaw and sold on the market as papain. The proteases may be designated as acid proteases, pepsin type enzyme or alkali protease, trypsin type enzyme. The mold enzymes are active in a neutral medium and may be a composition of both of the above types of enzymes encountered in the higher plant and animal cells.

HISTORICAL

Bitter discovered proteolytic enzymes in organisms liquefying gelatin. Fermi and Pampersi (1) poured the enzyme fluid on solid gelatin containing thymol and placed in a small diameter tube, to determine the proteolytic activity by observing the number of millimeters liquefied. They noted, however, that the time necessary for digestion was as important a factor as the amount liquefied. Geret and Hahn (2) noted the presence of albuminoids in juices of yeast cells as extracts of the cell. Tauret (3) was one of the first to establish the influence of the culture medium on enzyme production. Gamaleia (4) was able to confirm the work of these workers.

INDUSTRIAL USE

Proteolytic enzymes are used in the textile industry to remove protein substances from cotton goods and in the tanning industry to remove elastin from hides before tanning (5, 15). In baiting hides protease plus a deliming agent as ammonium chloride will remove the keratin (6, 17). After the hides are treated with dilute caustic the hairs may be loosed by use of such ezymes (7). The clarification of syrups of sediments that appear on standing has also been attempted. The Aspergilli have also been used in food preparation (14) for human consumption and in the preparation of chicken feeds.

NATURE OF PROTEASES

The mold proteases are active in a neutral medium as are trypsin and papain. The acid range for mold proteases is more limited than for pepsin, while the alkaline range is more limited than for papain. Fermi (8) finds the microbial proteases identical with trypsin and that they attack all proteins. De Waele and Vandevelde (9) suggest that the differences in bacterial proteases are differences of intensity only.

According to Malfitano (10) the protease of *Aspergillus niger* has the characteristics of a vegetable proteolytic enzyme for it resembles that of papaine and the proteolytic enzyme of malt. It acts on gelatin, nucleo-albumins, globulins and albummates, however, egg albumin is not digested and coagulated albumin is not attacked.

PRODUCTION OF PROTEASE

The amount of enzyme produced by certain cells depends as much on the substrate as on the given cell. Diehl (9) states that the enzymes are dependent upon the medium and differences in chemical constituents of the medium may account for development of new biological properties of microörganisms. Dox (11), however, found no new enzymes produced by variation in the culture medium but does observe stimulation in the production of particular enzymes already formed by the given organism. Young (12) finds protease production strongly stimulated by protein and followed by peptides, amides, ammonia salts, progressively weaker. The effect of the various media on the time of sporulation will effect the enzyme production (13). Protease is prepared by inoculating wheat bran with *Aspergillus oryzae*, and after growth, percolating water through the entire mass. The enzyme is taken up by the water and by repeated washings with the same water the concentration of the enzyme is raised. The last washing is done with fresh water to remove all extra-cellular enzyme but the intra-cellular enzyme remains.

Malfitano (10) used *Aspergillus niger* to produce protease. The quantity of protease depends on the mass of cells and the degree of maturity which they attain. The enzyme does not appear until the death of the cell.

Most workers have found wheat bran to be the best and cheapest medium on which to grow *Aspergillus oryzae*. Oshima and Church

(13) have used other media with good results with special note as to the physical condition of the ingredients.

Organisms that have been used commercially are: *Aspergillus sydowi*, *Aspergillus niger*, *Aspergillus terreus*, *Aspergillus oryzae*, *Aspergillus ochraceus* and related species.

QUANTITATIVE METHOD

Most methods used for quantitative estimation of protease are based fundamentally on the Fuld-Gross (13) method for trypsin. Oshima and Church (13) prepare a 0.5 per cent casein solution. Two grams of dry casein are mixed with 200 cc. of water and 50 cc. of N/10 sodium hydroxide and warmed to 70°C. After cooling it is neutralized with N/10 hydrochloric acid. Three cubic centimeters of N/10 sodium hydroxide are added and the whole made up to 500 cc. with distilled water. The casein was made by Hammarsten's method and the moisture estimated. Another solution is made of 4 parts of magnesium sulphate to 1 part of concentrated nitric acid.

A set of test tubes are arranged and 5 cc. of the casein solution is added to each tube respectively, followed by the addition of progressive amounts of the protease solution. The contents of the tubes are mixed and placed in a water bath at 40°C. for exactly one hour, at which time 0.5 cc. of the nitric acid solution is added to each tube. If the casein is undigested the solution is opaque. If water-clear the digestion is complete. The strength of the enzyme is determined by the amount of casein digested in a definite time at a given temperature. The proteolytic power is expressed on the basis of 100. If 0.025 cc. of original enzyme material digests 5 cc. of 0.5 per cent casein solution in one hour at 40°C. the proteolytic value is 100 units.

PRESERVATION OF ENZYME

The enzyme can be preserved for a period when dry and powdered but is rapidly destroyed when in solution. Some preservatives are toluol, chloroform, sodium fluoride, ether, phenol, thymol, oil of mustard and formaldehyde (12).

BACTERIAL PREPARATION

A seven day culture of *Escherichia ichthyosmia* (13) grown at room temperature in 2000 cc. of peptonized milk was saturated with thymol. After standing a period of ten days the liquid was filtered and

tested on sterile skimmed milk and sterile 2.5 per cent gelatin. The optimum pH value was from 6 to 8. A 6 per cent sodium chloride solution weakens and a 20 per cent solution inactivates the enzyme.

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CHAPTER 35

Tannase Production

For the production of tannic and gallic acids, the gall nuts, pathologic growths on oaks, sumacs and chestnuts, have been utilized commercially for many years, the gall nuts containing high concentrations of tannin compounds. Usually the nuts are ground and mixed with water and then left to a natural fermentation resulting in the formation of gallic and tannic acids.

Robinquet (1) attributed fermentation in gall nuts to a naturally contained ferment and believed that tannic acid was transformed by pectase. Van Tieghem (2) first isolated *Aspergillus niger* from a fermenting vat of gall nuts and studied its action in tannin fermentation. Fernbach (4) extracted the enzyme tannase from fungus grown in Raulins solution and Pottevin (5) did the same and also isolated it from *A. niger* when grown on Raulins solution with gallic acid but no sugar present.

The fermentation may be due to one or more of a limited number of fungi present on the nuts. Tannic acid is toxic to a large number of fungi at relatively low concentrations. Kundson (7) found that *Aspergillus flavus*, *Aspergillus oryzae* and *Aspergillus niger* and a few Penicilli only survived. Thom (8) has isolated *A. niger* and a Penicillium and one yeast from such a vat. The gall nuts used commercially for tan liquor production usually are imported from the orient or the "near east" and the *A. niger* is the predominating organism present on them.

Kundson (7) has reported extensive studies on the tannic acid fermentation with the idea of possibly improving the process by using pure culture controlled methods rather than the rule of thumb methods usually employed. He found that *A. niger* is a more vigorous fermenter than the Penicillia, and that fermentation in gall nut infusion is more rapid than in the synthesized solutions with tannic acid as the sole source of carbon (6).

Kundson found that the fungus, *A. niger*, would attack tannic acid, converting it to gallic acid and then utilizing it as a source of carbon. Other organic compounds in the gall nuts seemed to protect the gallic

acid and permit its accumulation. Cane sugar to 10 per cent if added to a 5.5 per cent gallic acid solution would spare the gallic acid. The fermentation will occur anaerobically, 1 mg. of mycelium transforming 2.706 grams of tannic acid in ten days. The enzyme is secreted both by the surface growth and the submerged mycelium.

Kundson found that for aerobic fermentation tannic acid plus sugar was most economical, while anaerobically tannic acid alone gave better results. In this experimental work he grew the fungi in a modified Richard's medium.

	grams
Potassium nitrate	1.00
Potassium dihydrogen phosphate	0.50
Magnesium sulphate	0.25
Water	1000 cc.

or a modified Czapeck fluid

	grams
Magnesium sulphate	0.50
Potassium dihydrogen phosphate	0.10
Potassium chloride	0.50
Sodium nitrate	2.00
Water	1000 cc.

He used 10 per cent cane sugar, tannic acid and gallic acid as carbon sources and inoculated by Hasselbring's method (6). Only two fungi tested by him were found to be able to use tannic acid, *A. niger* and *P. glaucus*. The *A. niger* first uses other organic compounds and after fructification secretes tannase which converts tannic to gallic acid which later is used as a source of carbon. *P. glaucus* uses gallic acid from the start so it gives lower yields.

In preparing experimental solutions of gall nuts 1800 grams of ground gall nuts were extracted with 3 liters of water for five days, drained and extracted with another 2 liters. After filtration these extracts were used with or without sugar addition. The nuts contain tannic and gallic acids, chlorophyl, starch, gums, sugar, protein and inorganic salts.

Tests for enzyme action were made as follows. Equal weights of pulverized mycelium, which had been collected on a crucible washed dried and weighed, were added to flasks containing dilute tannic acid solution, 0.5, 0.75 or 1 per cent. Two per cent toluene was added and

the flasks stoppered and incubated (time not stated) and then tested by Jean's method (3) for gallic acid.

Kundson found a progressive increase of tannase from the fungi with increasing addition of tannic acid to Czapek solution containing 10 per cent of sugar (7). With higher sugar concentrations tannase decreases. While no definite recommendations are made one gathers from reading Kundson's papers that in the preparation of tanning solutions from gall nuts it might be advisable to add up to 10 per cent of carbohydrate (possibly as molasses or as crude dextrose) to the water and to seed with spores from pure cultures of *Aspergillus niger*.

ENZYME TEST

Boiden (9) uses a test for enzyme activity by comparing the amount of enzyme solution necessary to coagulate milk.

1 cc. condensed milk.
4 per cent calcium chloride.
pH value 5.9 to 6.0.
0.2 cc. phosphate buffer.
Given amounts of enzyme added.
Total volume made up to 2.2 cc. with boiled enzyme solution.

A bacterial enzyme is determined after standing fifteen minutes at 60°C., while a pancreatic enzyme is determined after standing the same period of time at 35°C. The results may be compared with a standard enzyme solution.

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Section XI

BIOLOGICAL PROCESSES IN INDUSTRY

CHAPTER 36

The Development of New Processes

It has long been recognized that certain organisms play an active part in the production of certain chemical substances used in industry. Some of these processes have been investigated in considerable detail and in some instances specific organisms have been isolated and studied, but in a number of others no such studies have been made. While the rôle of the bacteria has been recognized they have not been isolated and worked with in pure culture so as to understand clearly the effect of various modifications in process controls or the reasons for occasional failures to obtain satisfactory yields.

Many of these processes have been investigated primarily from the chemical or physical standpoints, the bacterial action, and the conditions essential for maximum activity and for continued adequate functioning have been little if at all understood. This has resulted in great variations in yields and at times in unexplained complete failures to produce.

Such biological products as acetic and lactic acid and ethyl alcohol have long been studied from this standpoint, the first not yet usually being conducted as a pure culture fermentation and the last often not properly controlled.

In a number of instances patented processes have been discussed in much detail because we felt that the problems met with in these processes would be of interest to others attempting to develop new industrial biological processes along other lines.

In many ways the problems of industrial biology differ widely from those of medical bacteriology and even, in a less degree, from those of other specialized bacteriological fields such as soil, food, or dairy bacteriology. In industrial bacteriology we study beneficial organisms, endeavoring to develop them to their point of maximum activity.

While pure culture work is essential in medical bacteriology, in industrial fermentations it may or may not be so. At times almost equally good results may be obtained from the use of predominating cultures and in some instances, as in vinegar production, two unrelated organisms may be necessary for a complete reaction.

Industrial bacteriology is of no practical avail until fermentations can be measured in hundreds or thousands of gallons. This mass culture work increases enormously the difficulties of maintaining pure cultures and makes it necessary many times to be satisfied with predominating culture fermentations.

The medical bacteriologist studies toxins and antibodies, and may use either to advantage. The industrial bacteriologist is vitally interested in enzymes and must educate his organisms by changes in environment and nutrients so that they may produce a maximum amount of the specific enzyme desired. Where organisms produce a variety of enzymes, as is particularly the case with certain fungi used in industry, it is often found that particular strains of what is apparently a single species may differ widely in the predominance of the various enzymes produced. While it is generally accepted that one cannot educate organisms to produce an enzyme not formerly produced by it, yet one can often increase very greatly the amount of an enzyme produced in very small amount by others of the same species. In general cultivation on substrates attacked by the desired enzyme will stimulate its production, while cultivation on substrates high in the end products of the specific enzyme action will decrease its production. An increase in a specific enzyme activity brought about by such education may persist through several generations so that the education of seeding cultures in the laboratory may serve to materially increase activity in massive production from vat cultures in the plant.

In industrial fermentation we are primarily interested in the end products from definite substrates and seek to employ organisms giving the highest yields regardless of their specific classification. Where organisms are studied for their enzymatic activities, as these may vary widely in different strains of the one species, it not infrequently happens that the same organism is described and used by different workers and even patented under different names, as is undoubtedly the case with those organisms used to produce butyl alcohol and the lactic ferments.

We have endeavored to describe methods for isolation of organisms from specific sources, but many of the organisms considered in the specific fermentations discussed are obtainable in pure culture from the American Type Collection at the McCormick Institute in Chicago (3). We would caution those obtaining cultures from this source,

however, for while they may be and usually are pure cultures giving normal growth reactions and producing normal enzymes for the species, yet they may have been subcultured so long at infrequent intervals on media suitable merely to keep them alive that they may have lost much of their power to produce in appreciable quantity the specific enzymes desired. We would strongly advise their frequent subculturing on carefully selected media under favorable conditions before attempting to study yields quantitatively. We would also advise anyone entering on industrial fermentation studies to attempt to isolate their own cultures from natural sources, using the type cultures for comparison. One may in this way obtain a new strain definitely more active than the stock organism.

In this work we have considered reactions produced by bacteria, yeasts, and molds. The former have long been used and their activities recognized but the average bacteriologist has generally neglected the yeasts and molds, usually considering them as pests and contaminations.

The alcoholic fermentation industries have studied the yeast fermentations in great detail but even they, in this country at least, have neglected the molds.

In the description of bacterial processes we have adhered closely to Bergey's *Manual* (5). For the Aspergilli and Penicillia we have relied on Thom and Church (2) and Thom (4). The industrial adaptability of the Aspergilli has been ably presented by Dr. Thom, whom we quote.

> "Industrial use of Aspergilli has been restricted to a small number of species. A mold fermentation to be successful commercially must either produce a product of such price as to warrant expert service with the maintenance of pure cultures or must be based upon an organism capable of vigorous growth under a wide range of conditions. Such an organism must grow rapidly enough to overgrow and swamp such competing species as may be able to survive the conditions of culture or must be tolerant of such concentrations of a suitable inhibiting agent as will automatically restrict the flora of the fermenting mass." (2)

Aspergilli usually develop on the surface of solutions only and so fermentations must be conducted in large flat pans or shallow trays increasing the difficulty of maintaining pure cultures. In spite of this several species have been used successfully in plant scale fermentations. Aspergilli produce a number of enzymes but only a few of

them have been used to advantage in production. Carbohydrate splitting enzymes producing organic acids and alcohol tannase, forming gallic acid from tannin, and protease about cover the list. These fermentations have been considered in detail.

For yeast studies we have drawn upon Lafar (1) and a number of other sources, as noted in the chapter bibliographies. We have culled freely and widely from the periodical and monograph literature, endeavoring to present a logical development of the work of others brought together in one place rather than to stress our own work and views.

We have considered all or most of the biologic processes known to be feasible industrially and some about whose feasibility we have considerable doubt but which are interesting and suggestive. We feel, however, that the field of industrial fermentation is a growing one and that there are other raw materials that may be employed in such processes. We feel that the list of usable organisms will be materially enlarged in the future and that many more useful end products may be obtainable by these means, some as economically or more economically than at present prepared by purely chemical processes, and others not now available in commercial quantities.

We hope that the study of the processes discussed in this work may be an aid and a stimulus to those contemplating the development of new types of industrial fermentations, and that they may be a help to many already working on some of the processes here considered.

There are many factors which must be considered before deciding to undertake a fermentation process on an undustrial scale.

ORGANISMS

Will the organism, if cultivated under uniform and favorable conditions, give constant and fairly uniform yields of the desired end product?

Is it easily cultivatable on fairly simple inexpensive media?

Is it aerobic, facultative, microaerophilic or anaerobic? These factors will influence the conditions of fermentation and the shape and size of the fermentation vessels. For aerobic fermentations one must have large surface areas with comparatively little depth or must arrange to aerate deeper vessels by stirring or by bubbling sterile air through. An actively gassing fermentation if open to the air automatically stirs itself but if it is desired to collect the fermentation

gases the vessel must be covered and so exclude air. With microaerophilic or anaerobic organisms deep vessels are an advantage, and with gassy fermentations and covered vessels after the fermentation is well under way the active gas evolution serves to exclude air.

Is the organism easily obtainable from natural sources so that if for any reason the original culture is lost or loses its activity it may be replaced?

Is the organism the only one available that will produce the desired result or will other related or non-related organisms serve?

Are pure culture fermentations necessary or advisable, will predominating cultures suffice or are mixed culture or successive culture processed required?

SUBSTRATE

Is there available an abundant easily obtainable and easily handled cheap raw material, or may a number of raw materials be used in case the supply of the selected one is lessened?

Can the raw material be fermented in its natural state, or must it be hydrolyzed by physical, chemical or biological means before it is available and will such pre-treatment add materially to the cost of the process?

Can the raw material be used in reasonably concentrated form, must it be excessively diluted before use, or concentrated considerably to make the process worth while? The removal of excess water before or after fermentation may be expensive and it does not pay to have to transport excess water over long distances.

PROCESS

Is the process of pre-treatment, fermentation, and recovery very complicated, expensive, or with difficultly maintainable conditions?

Is the time necessary to obtain economically satisfactory yields excessively long? Long continued fermentations must either automatically limit the output of a plant or necessitate inordinately larger installations.

Is the end product desired easily recoverable in a sufficiently pure and sufficiently concentrated state to be marketable economically or is elaborate expensive equipment or process necessary?

Is there only one marketable end product obtainable or are there more or less valuable by-products to be had?

Has the residue, liquid, or solid sludge any marketable value or if not can it be easily and cheaply disposed of?

The failure to give due consideration to any one of the above factors may make the difference between a profitable worthwhile industry or a financial loss and disappointment.

DEVELOPMENT

Having decided that a new process may be developed, and has possibilities, much preliminary work remains to be done. If the process seems feasible when the organisms are cultivated on a laboratory scale in liter or multiple liter fermentations, attempt to duplicate fermentations of 50- or 100-gallon mashes or washes, and if that is successful then go to small plant scale production. It should take several years to develop a new process to the point of establishing a profitable industry.

Where maintenance of pure culture fermentation throughout is essential the difficulties of operation are increased greatly, and so is the cost of installation.

TABLE 19

PATHOGENIC AND NON-PATHOGENIC MICROORGANISMS

	NOT PATHOGENIC	PATHOGENIC FOR				
		Man	Animal	Plant	Man and Animal	Total
Number of species.....	840	80	66	104	30	1120
Percentage.....	75	7.1	5.9	9.2	2.7	—

DESCRIBED SPECIES OF BACTERIA

There are 1120 species of microörganisms listed in Dr. Bergey's *Manual of Determinative Bacteriology*. Of this number 840 or 75 per cent are non-pathogenic. Dr. Diehm has prepared a chart showing the total and percentage of pathogenic and non-pathogenic microorganisms.

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CHAPTER 37

The Relation of Patent Law to Biological Processes[1]

INTRODUCTION

The microörganisms appear to have gone into business. Imperceptibly their myriads have crept into industry, where they are nourished and pampered for the work that they do. The beneficial results of microbial activity are being given recognition and the unicellular organisms are regarded as the allies of man. This is not surprising, however, when it is remembered that the science of microbiology had its origin in the fermentation industry of France. It was born of Industry.

The field of pathogenic bacteriology has undergone more spectacular development than the field of industrial processes, so that it has far overshadowed the slowly developing field of biological processes in industry. But we note that of the thousand and more species of bacteria alone, less than 25 per cent are pathogenic. A vast majority of this one group of unicellular organisms, the bacteria, not only are beneficial but are absolutely essential to higher plant and animal life. The practical application of bacteria, yeasts and molds in industry is again being exploited, with the realization that few workmen toil so faithfully and with such startling results.

In the protoplasm of these minute cells are contained all of the enzymes necessary to supply them with energy for their growth and multiplication. Each cell is a perfect little chemical factory, producing directly by intramolecular rearrangement or indirectly by extracellular enzymes the end products of a given specific fermentation, employing the term fermentation in its present broad sense.

What protection have these microscopic factories in industry, and commerce, or better what protection have their promoters, the scientist and the industrialist? Will patent law protect investments in this new factor in industry? Can any good patent at all come out of the chaos of pure science and natural processes involved?

[1] This entire chapter has been written by Mr. J. Howard Flint, patent lawyer, Farmers Bank Building, Pittsburgh, Pa.

AUTHORITY FOR PATENTS

The patent laws of the United States are founded on the Federal Constitution and to some extent on analogous provisions of earlier laws. To Congress was delegated the duty of "promoting the progress of science and the useful arts by securing for limited times to authors and inventors the exclusive right to their respective writing and discoveries." Hence, to the extent that science and the useful arts are promoted by the commercialization of microbiology, the foundation for patent protection has been laid.

DUTIES OF INVENTOR

The law imposes the burden on the inventor to define what is new about his invention; it is incumbent on him to apply for the patent, to explain what his invention is and how to duplicate it.

The public is to be taught what it is that is alleged to promote science and the useful arts and how to use or duplicate the new invention or discovery without having itself to resort to experiment.

FIRST STEP FOR PROTECTION

Even in such a highly technical field as microbiology, with the scientific worker as familiar as he is with the "prior art," it should not be assumed that a given step is new in a patentable sense. It is important to have the records surveyed so that what is new may be distinguished sharply from what is old. The proper time for this, curiously enough, is before the contemplated commercial process is completed. In this "prior art" survey sould be included the United States patents bearing on the particular subject, and it may well include the patents of those foreign countries that are active in this field, as England, France and Germany. The general literature of the subject also should be considered as well as such knowledge as is at hand concerning prior public use of the type of microbial process or product in question. This preliminary information is not exhaustive; but, armed with it, subsequent research will profit more and the patent situation will be developed on a better basis.

PATENT CONSIDERATIONS, TECHNICAL

The patent expert considers this "prior art," in conjunction with the particular industrial development being studied, with respect not only to anticipation but to patentable equivalents and with relative

breadth of claims in mind. He enquires whether the proper protection may be obtained in one patent or whether a group of related patents is better; whether there are incidental steps to be claimed and new by-products to be protected, or new compositions of matter, or a new type of apparatus, or a new process. He also will consider whether patent protection should be supplemented by trade marks.

In general, the possible subject of protection permitted by the patent statutes of the United States have been first, new processes; secondly, new apparatus; thirdly, new articles of manufacture; fourth, new compositions of matter; and finally new improvements in these four basic classes. Of course these must be useful, but that generally may be assumed.

The improvements may be but slight. The test is not whether a great stride has been taken; the test rather is whether it is something other than what the technical expert in this field would have expected normally from his knowledge of the prior art and without the suggestion or teaching of the proposed invention. The questions of basic patents and breadth of claims also are important considerations. Their part in a given commercial picture can better be analyzed by the expert at the proper time. The slightest specific advance may well make the difference between successful operation or continued and unsuccessful experimentation.

PLANT PATENTS

During the latter part of May, 1930, the patent statutes were amended to add a fifth subject of protection. They now provide that any person "who has invented or discovered and asexually reproduced any distinct and new variety of plant, other than a tuber propogated plant . . . . may obtain a patent therefor," "including in the case of a plant patent the exclusive right to asexually reproduce the plant."

The reaches of this innovation in patent law remain to be seen. The constitutionality of the provisions will have to be determined and exact interpretation placed on the terms employed. But to industrial microbiology for the present this provision appears to be an open door of vital importance.

TAXONOMY OF PLANTS

Plants are divided into four large groups, the Thallophyta, or thallus plants, the Bryophyta or moss plants, the Pteridophyta or fern plants, and the Spermatophyta or seed plants.

No restriction appears in the statute against patenting the asexual reproduction of new and distinct varieties in any one of these four groups. In the field of microbiology, alone, the ramifications of possible protection are exceedingly vast, even when limiting it to the one-celled organisms, and excluding the protozoa, or the one-celled organisms of the animal kingdom. It is by use of the unicellular plants that industrial microbiology today is taking its place in the commercial sun.

The three major classes of the one-celled plants, the Schizomycetes or bacteria, the Saccharomycetes or yeasts, and the Hyphomycetes or molds constitute the foundation for the development of industrial fermentation. On this fundamental basis, for patents directed to these microscopic plants, the industrialist may protect the new and distinct species utilized in commercial processes. It seems reasonable to read from the present statutes that mutants of bacteria, yeasts and molds now may be the subject of patent protection. The possibilities further appear from the express statutory provision that the description required need be only "as complete as is reasonably possible." The proper course undoubtedly is to file the required patent applications without waiting for any final clarifications of the new statutes, as soon as the technical data are at hand.

BIOLOGICAL PROCESS PATENTS

The patentability of processes deserves considerable attention for microbial processes unquestionably can be the basis for patent protection even though something of the action of living organisms is involved.

The factors that may lead to patentably new effects first would require consideration of the organisms that act. These may not necessarily be new, but the definition of the invention would point out what type is involved. The expert should know how broadly generic classifications may be employed. The technical classifications, with which the bacteriologist is familiar, naturally would supply the language and define the breadth of the patent claim.

The raw material constitutes another factor of the process. Such

raw material might itself be old, but its use in combination with the particular organisms may be the cause of new results.

There is also a compounded type of raw material which as a combination or composition of matter may be new; something that could be sold as an article of commerce to supply a desired environment or nutrient medium. Such compositions present the possibility of being patentable and further of being protected under the trade mark laws.

A third factor of the bacteriological process, possibly the most fruitful source of protection, comprises the conditions of operation. Examples that suggest themselves are a proper change of pressure at a given stage, a definite pH range of acidity or alkalinity conditions, a temperature control of a definite sort, or an alteration of the fundamental fluid medium in which the bacteria operate. The possible permutations and combinations of these factors are most extensive. They may indeed be such as normally are investigated by the scientist, but the result of teaching what definite conditions yield a definite sort of result generally would justify the grant of patents commensurate in scope with the discovery.

Some confusion arises at times from distinctions between discovery and invention. Patentable subject matter is such as expresses something operative or tangible as distinct from the abstract conception that characterizes a discovery. The purpose of the patent laws is to put discoveries to work, to teach the public how to operate with given materials and principles. If one obtains better products, exceptional yields, or new materials some new factor must be involved. A patent analysis endeavors to define the situation in terms of the useful results as well as the new.

SECRET PROCESSES

The alternative of patenting is the secret process. The issue must be decided at the outset, for once a process has been operated in secret and then finally patented, such patent might be invalidated simply on a showing of the prior secret practice.

There are always dangers to the secret process. Business experience for centuries has shown that trusted employees prove to be unfaithful, and that competitors find various ways to learn the secrets. In our modern day this latter especially would be true, for the competitor also commands a choice of scientifically trained men. And when this competitor has worked out a given process *de novo* he

probably will patent it. He then dominates and may exclude the one who is attempting to carry on in secret. Another disadvantage to the business endeavoring to operate without patent protection is that the inventor may die and afterwards it may become desirable to patent the invention. However, that may be too late for with but limited exception a patent application must be made and sworn to by the inventor.

PROCEDURE TO OBTAIN PATENT

Primarily the procedure is to apply to the Patent Office for patent, accompanying the petition by a clear explanation of the invention and the best mode of carrying it out, finally distinctly pointing out what the applicant claims to be the novel features of the invention. The inventor or inventors must make the application, but later may assign it. This apparently simple procedure has become very complicated and is built up on an elaborate structure of technical decisions and rulings. Cooperation between the patent lawyer, the industrial promoter and the bacteriologist is essential if patent claims of dominating scope are to be wrought out before the Patent Office with such finesse as will survive the subsequent scrutiny of a court. It must be borne in mind that a patent is a legal document and that the most careful attention and wording must be employed at every stage of its development. It is a weapon for legal warding off of one's competitor, hence it must be fashioned to anticipate his counter-attacks.

Once the aplication is filed, protection of a sort is initiated but no generalization can be made as to how far nor when the invention may be commercialized. Practice at that stage depends on the facts of a given situation, but it may not be at all necessary to wait until a patent actually issues. Indeed delay in issuing the patent may in effect add to the seventeen years of monopoly proper. One patent application was kept pending thirty-five years before the patent actually issued.

Questions that arise between employers and the employee entitled to patent his invention will occur under the widest variety of circumstances. From the mere fact that the inventor is an employee it by no means follows that the employer is entitled to own the invention nor even to practice it. Unless an employee clearly has contracted away his rights or has made extensive use of his employer's facilities to develop the invention, the courts are reluctant to diminish the

inventor's patent rights. The arrangement of contracts and licenses are really part of the legal situation created by the parties; hence as careful attention should be paid to these documents as to the patent itself.

Assigments similarly are expressed by legal documents at the time an invention is sold or mortgaged. An assignment of the invention may be made before the patent itself is granted. The parties should make it clear whether the whole invention is being sold, the incidental parts as well as the general combination, and whether the sale is confined to the patent of a particular country. This is important because in some foreign countries the application need not necessarily be made by the inventor but may be made by the assignee owner.

This ability to assign a patent may meet the ethical quandry in which the scientist often finds himself. For one reason or another he may consider that it is improper for him to impose a monopoly on the public. This involves some misconception of the situation. The patentee of the United States is not required to use his invention, but may throw it open to the public just as freely as with something unpatented. However with the patent he may not only provide reimbursement for his research, but may protect the public itself from exploitation by those economic powers that be, those few who have the extensive facilities to manufacture or to sell the new product of science at their own price. The patentee in granting permission to manufacture or sell may impose such terms as he chooses, such as to price, purity of product and distribution of a sort that the public really may benefit. Of late, patents covering such scientific results have been assigned to trustees or to corporations designed to administer the patents for the benefits designated. Perhaps an outstanding merit of this procedure is to prevent patenting by an imitator or subsequent rediscoverer who might not be actuated by high ethical ideals.

The patent system exists as a part of the economic, legal and even scientific organization of the day. Technicalities exist in the system because they are almost inevitable to any orderly meeting of the many situations involved. The system however can render incalculable service and it renders most to those who approach with the fullest measure of professional ability, business acumen and technical aid and knowledge.

Section XII

BACTERIOLOGICAL SURVEY

DESCRIPTION OF THE CAUSATIVE AGENT USED IN BIOLOGICAL PROCESSES

DESCRIPTIVE CHART

ENDORSED BY SOCIETY OF AMERICAN BACTERIOLOGISTS

Prepared by Committee on Bacteriological Technic

Name of organism.....

Source.....

Date of isolation.....

Culture number.....

Studied by.....

Invigoration of Culture.....

Date..... Series number.....

Medium used.....

Temperature.....°C. Number of transfers.....

Length of each incubation.....

MORPHOLOGY

UNDERSCORE REQUIRED TERMS	SKETCHES
VEGETATIVE CELLS, Medium used..... temp.....age.....days. Form, spheres, short rods, long rods, filaments, commas, short spirals, long spirals, curved. Arrangement, single, pairs, chains, fours, clusters, cubical packets. Limits of Size..... Size of Majority..... Ends, rounded, truncate, concave. CAPSULES, present on..... How stained.....	
SPORANGIA, present, absent. Medium used..... temp.....age.....days. Form, elliptical, short rods, spindled, clavate, drumsticks. Limits of Size..... Size of Majority..... ENDOSPORES, present, absent. Location of Endospores, central, polar. Form, spherical, elliptical, elongated. Limits of Size..... Size of Majority..... Wall, thick, thin. Sporangium wall, adherent, not adherent.	
MOTILITY. In broth..... On agar..... FLAGELLA, No..... Attachment, polar, bipolar, peritrichiate. How stained.....	
IRREGULAR FORMS. Present on.....in.....days at.....°C. Form, spindled, cuneate, filamentous, branched, or.....	
STAINING REACTIONS. Gram..... Acid fast..... Special stains.....	

CULTURAL CHARACTERISTICS

	UNDERSCORE REQUIRED TERMS	SKETCHES
Agar Stroke Incubation Temperature .....°C Age .....d	Growth, scanty, moderate, abundant, none. Form of growth, filiform, echinulate, beaded, spreading, arborescent, rhizoid. Elevation of growth, flat, effuse, raised, convex. Lustre, glistening, dull. Topography, smooth, contoured, rugose. Optical Characters, opaque, translucent, opalescent, iridescent. Chromogenesis.....Photogenic. Fluorescent. Odor, absent, decided, resembling..... Consistency, butyrous, viscid, membranous, brittle. Medium, grayed, browned, reddened, blued, greened.	
Gelatin Stab Temperature .....°C Age .....d	Growth, uniform, best at top, best at bottom. Line of puncture, filiform, beaded, papillate, villous, arborescent. Liquefaction, none, crateriform, napiform, infundibuliform, saccate, stratiform; begins in.....d; complete in.....d. Depth of liquefaction in tube of 10 mm. diameter evenly inoculated at 20°C. for 30 days.....mm. Medium, fluorescent, browned.	
..... Medium (solid) Temperature .....°C Age .....d		
Nutrient Broth	Surface growth, ring, pellicle, flocculent, membranous, none. Clouding, slight, moderate, strong,	

CULTURAL CHARACTERISTICS—*Concluded*

UNDERSCORE REQUIRED TERMS		SKETCHES
Temperature .....°C Age .....d	transient, persistent, none, fluid turbid. Odor, absent, decided, resembling..... ..... Sediment, compact, flocculent, granular, flaky, viscid on agitation, abundant, scant, none.	
..... Medium (liquid) Temperature .....°C Age .....d		
..... Medium Temperature .....°C Age .....d		
Agar Colonies Temperature .....°C Age .....d	Growth, slow, rapid. Form, punctiform, circular, irregular, mycelioid, filamentous, rhizoid. Surface, smooth, rough, concentrically ringed, radiate. Elevation, flat, effuse, raised, convex, pulvinate, umbonate. Edge, entire, undulate, lobate, erose, filamentous, curled. Internal structure, amorphous, finely-, coarsely-granular, filamentous, curled, concentric.	
Gelatin Colonies Temperature .....°C Age .....d	Growth, slow, rapid. Form, punctiform, circular, irregular, mycelioid, filamentous. Elevation, flat, raised, convex, pulvinate, crateriform (liquefying). Edge, entire, undulate, lobate, erose, filamentous, floccose, curled. Liquefaction, cup, saucer, spreading. Internal structure, amorphous, finely-, coarsely-granular, filamentous, curled, concentric.	

PHYSIOLOGY

Relation to Oxygen:

Method used.....

Medium.....Temperature.....°C.

Aerobic growth; absent, present, better than aerobic growth.

Anaerobic growth; absent, occurs in presence of dextrose, of sucrose, of lactose, of nitrate; better than aerobic growth.

Diastatic Action:

Breadth of clear zone on starch agar plates in.....

days.....Additional data.....

Milk:

Temperature.....°C.

Reaction	1 day....	2 days....	4 days....	7 days....	10 days.....
Acid curd	1 day....	2 days....	4 days....	7 days....	10 days.....
Rennet curd	1 day....	2 days....	4 days....	7 days....	10 days.....
Peptonization	1 day....	2 days....	4 days....	7 days....	10 days.....

Reduction of litmus in....days; of methylene blue in....days. Influence of indicator on growth.....

Nitrate Reduction:

Medium.....Temperature.....°C.

Nitrite:	1 day....	2 days....	4 days....	7 days....	10 days.....
Gas:	1 day....	2 days....	4 days....	7 days....	10 days.....

Fermentation:

Medium containing.....	DEXTROSE	LACTOSE	GLYCERIN	SUCROSE	ETC.	ETC.	
.....							
.....							
Temperature.....°C.							
Gas.....							
First appearance of acid.....							
First appearance of alkali.....							
Reaction after.....days.....							
Reaction after.....days.....							
Maximum H-ion concentration.....							

Temperature Relations:

Optimum temperature for growth.....°C.

Maximum temperature for growth.....°C.

Minimum temperature for growth.....°C.

Chromogenesis:

Nutrient broth.....

Nutrient gelatin.....

Nutrient agar.....

Potato.....

Production of Indol:

Medium.....

Indol absent, present in.....days

Production of Hydrogen Sulfide:

Medium.....

Hydrogen sulfide absent, present in.....days

INDEX NO.*.....

BRIEF CHARACTERIZATION

As each of the following characteristics is determined, indicate in proper marginal square by means of figure, as designated below:

PRIMARY CHARACTERISTICS	Microscopic Features	Form: 1, streptococci; 2, diplococci; 3, micrococci; 4, sarcinæ; 5, rods; 6, commas; 7, spirals; 8, branched rods; 9, filamentous	
		Spores: 1, central; 2, polar; 3, absent	
		Flagella: 1, peritrichic; 2, polar; 3, absent	
		Gram stain: 1, positive; 2, negative	
	Miscellaneous Biochemical Reactions	Pathogenicity, etc.: 1, for man; 2, for animals; 3, for plants; 4, parasitic but not pathogenic; 5, saprophytic; 6, autotrophic	
		Relation to oxygen: 1, strict aerobe; 2, facultative anaerobe; 3, strict anaerobe	
		Gelatin liquefaction: 1, positive; 2, negative	
		In nitrate media: 1, nitrite and gas; 2, nitrite but no gas; 3, neither nitrite nor gas	
		Chromogenesis: 1, fluorescent; 2, violet; 3, blue; 4, green; 5, yellow; 6, orange; 7, red; 8, brown; 9, pink; 0, none	
	Carbohydrate Reactions	Diastatic action: 1, positive; 2, negative	
		From dextrose: 1, acid and gas; 2, acid without gas; 3, no acid	
		From lactose: 1, acid and gas; 2, acid without gas; 3, no acid	
		From sucrose: 1, acid and gas; 2, acid without gas; 3, no acid	
SECONDARY CHARACTERISTICS	Vegetative Cells	Diameter: 1, under 0.5μ; 2, between 0.5μ and 1 μ; 3, over 1μ	
		Length: 1, less than 2 diameters; 2, more than 2 diameters	
		Chains (4 or more cells): 1, present; 2, absent	
		Capsules: 1, present; 2, absent	

BRIEF CHARACTERIZATION—*Concluded*

Secondary Characteristics	Spores		Shape: 1, round; 2, oval to cylindrical	___
			Diameter: 1, less than diameter of rod; 2, greater than diameter of rod	
	Cultural Features	Agar Stroke	Abundance: 1, abundant; 2, moderate; 3, slight; 4, absent	
			Lustre: 1, glistening; 2, dull	___
			Surface: 1, smooth; 2, contoured; 3, rugose	___
			Agar colonies: 1, punctiform; 2, round (over 1 mm. diameter); 3, rhizoid; 4, filamentous; 5, curled	
			Gelatin colonies: 1, punctiform; 2, round (over 1 mm.); 3, irregular; 4, filamentous	
		Milk	Acid: 1, sufficient for curdling; 2, insufficient for curdling; 3, no acid	
			Rennet curd: 1, present; 2, absent	___
			Peptonization: 1, present; 2, absent	

* Recording the "Index Number" here is optional; but its use will be found convenient if the charts are to be filed according to the salient characteristics of the organisms. The Index Number consists of the first thirteen figures from the margin (primary characteristics) copied down in the order of their occurrence in the margin, placing a dash wherever a heavy rule occurs in the margin. Thus, B. coli belongs to the group 5312–41220–1111.

TABULATION OF MICROÖRGANISMS USED IN INDUSTRY

SCHIZOMYCETES

Order: Eubacteriales		
Family: Nitrobacteriaceae		
Tribe: Nitrobacteriae		
Genus: Nitrosomonas		
Nitrosococcus	Fertilizer production	Chapter 23
Nitrobacter		
Acetobacter	Manufacture of acetic acid and vinegar	Chapter 2
A. xilinum	Artificial leather	Chapter 15
	Food product	Chapter 27
Genus: Thiobacillus		
Tribe: Azotobacterieae		
Genus: Azotobacter	Fertilizer production	Chapter 23
Genus: Rhizobium		
Family: Coccaceae		
Tribe: Streprococceae		
Genus: Streptococcus		
S. lactis	Manufacture of lactic acid	Chapter 6
Tribe: Micrococceae		
Genus: Micrococcus		
M. phosphorescens	Food product	Chapter 27
Family: Bacteriaceae		
Tribe: Chromobacterieae		
Genus: Pseudomonas		
Ps. fluorescens	Desizing	Chapter 14
Tribe: Cellulomonadeae		
Genus: Cellumonas	Cellulose decomposition	Chapter 16, 23
Tribe: Achromobacterieae		
Genus: Achromobacter	Fertilizer production	Chapter 23
Tribe: Erwineae		
Genus: Erwinia		
E. carotovorus	Pectinase production	Chapter 33
Tribe: Lactobacilleae		
Genus: Lactobacillus	Ensilage, butyric acid	Chapter 18, 3
L. bulgaricus	Manufacture of lactic acid	Chapter 6
L. leichmanni	Manufacture of food prod.	Chapter 27
L. acidophilus	Manufacture of lactic acid	Chapter 6, 27

L. pentosus	Fermentation of xylose	Chapter 19
L. arabinosus		
L. delbruckii	Manufacture of lactic acid	Chapter 6
	Manufacture of food product	Chapter 27
	Mobilization of vegetable oil	Chapter 22
L. helviticum (B. casei)	Manufacture of lactic acid	Chapter 6
	Manufacture of cheese	Chapter 27
L. soya	Manufacture of food product	Chapter 27
L. pentoaceticus	Fermentation of xylose	Chapter 19
Tribe: Propionibacterieae		
Genus: Propionibacter		
P. shermanii	Manufacture of propionic acid	Chapter 7
Tribe: Bacterieae		
Genus: Escherichia		
E. acidilactici	Ensilage	Chapter 18
Genus: Proteus		
P. vulgaris	Manufacture of propionic acid	Chapter 7
Genus: Salmonella	Rat poison	Chapter 25
Genus: Shigella		
S. alkaligines	Manufacture of propionic acid	Chapter 7
Family: Bacillaceae		
Genus: Bacillus		
B. subtilis	Butyric acid production	Chapter 3,
	Bread making	14, 32, 27
B. mycoides	Bread making, proteolysis	Chapter 27, 14
B. vulgatus	Xylose fermentation	Chapter 19
B. mesentericus	Desizing, amylase production	Chapter 14, 32, 27
Genus:		
Clostridium	Cellulose fermentation	Chapter 16, 17
C. butyricum	Butyric acid, butyl alcohol, etc.	Chapter 3, 9, 8, 31
C. histolyticum	Desizing	Chapter 14
C. sporogenes	Desizing	Chapter 14
C. felsinus	Retting	Chapter 17
Order: Actinomycetales		
Family: Actinomycetaceae		
Genus: Actinomyces	Cellulose decomposition, etc.	Chapter 16, 23
Family: Mycobacteriaceae	Cellulose decomposition	Chapter 16
Genus: Cytophaga		
Genus: Cellvibrio		
Genus: Cellfalcicula		
Family: Chlamydobacteriales	Fertilizer production	Chapter 23

Order: Chlamydobacteriales		
Family: Beggiatoaceae		
Genus: Thiothrix		
T. tenuis	Butyl alcohol and acetone	Chapter 9

ORGANISMS NOT LISTED IN BERGEY'S MANUAL

Bacillus invertenti lactici	Lactic acid production	Chapter 6
Bacillus invertenti acetici		
Bacillus technis (possibly a variety of C. butyricum)	Butyl and iso-propyl alcohol	Chapter 10
Clostridium acetoethylicum	Acetone and ethyl alcohol	Chapter 13
Aerobacter sorbose	Xylose fermentation	Chapter 19
Aerobacter foeni	Xylose fermentation	
B. herbicula aurum	Xylose fermentation	

SACCHAROMYCETES

Genus: Saccharomyces		
S. cerevisiae	Ethyl alcohol, food products	Chapter 11, 27, 9
S. elipisoideus	Ethyl alcohol	Chapter 11
Genus: Torulae		
T. communis	Food production	Chapter 27
	Sugar preservation	Chapter 20, 27
Genus: Monila		
M. niger	Sugar preservation	Chapter 20
M. fusca	Sugar preservation	Chapter 20
Genus: Oidia		
O. lactis	Food production	Chapter 27
O. asteroides	Food production	Chapter 27

THE HYPHOMYCETES

Genus: Aspergillus		
A. niger	Citric acid production	Chapter 4
	Tannase production	Chapter 35
	Amylase production	Chapter 32
	Protease production	Chapter 34
A. flavus oryzae	Tannase production	Chapter 35
	Amylase production	Chapter 32
	Food preparation	Chapter 27
A. wenti	Tannase production	Chapter 35
A. oryzae	Butyl alcohol and acetone	Chapter 9
A. sydowi	Protease production	Chapter 34
A. tertius	Protease production	Chapter 34
A. ochraceus	Protease production	Chapter 34
A. flavus	Tannase production	Chapter 35
Sterigmatocystus niger (A. niger)	Citric acid production	Chapter 4

Genus: Penicillia		
P. luteum purpurogenum var. rubrisclerotium	Gluconic acid production	Chapter 5
P. citrinum	Gluconic acid production	Chapter 5
P. divaricatum	Gluconic acid production	Chapter 5
P. glaucum	Amylase production	Chapter 32
Citromyces pfefferianus	Citric acid production	Chapter 4
Citromyces glaber	Citric acid production	Chapter 4
Genus: Rhizobium		
R. tritici	Pectinase production	Chapter 33
R. oryzae	Food products	Chapter 27
Genus: Mucor		
M. rouxii	Amylase production	Chapter 32, 27
M. oryzae	Food products	Chapter 27

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